

# The role of the cholecystokinin 2 receptor in cancer

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## Abstract

The gastrointestinal (GI) hormone, gastrin, promotes cancer progression and its down-regulation has been linked to reduced cancer stem cell numbers. Gastrin acts through the cholecystikinin-2 receptor (CCK-2R) and its biological effects are blocked by CCK-2R inhibitors. We investigated the regulation of the CCK-2R and its potential role in promoting survival of cancer stem cells (CSC).

A panel of cancer cell-lines, including GI, glioblastoma and lung, with CCK-2R-transfected cells as a positive control, were grown either as monolayers, or, to provide a 3D *in vitro* tumour model, as spheres. Linear-after-the-Exponential (LATE)-PCR was used to quantify CCK-2R gene expression and this was validated using siRNAs. Flow cytometry was used to investigate receptor protein expression. Activity of CCK-2R promoter reporters was quantified using luciferase assays.

LATE-PCR for CCK-2R gene expression is 10,000-fold more sensitive than the Taqman-based assay, and provides a highly precise method for detection of genes which have important biological functions but low expression. This assay showed that primary non-small-cell lung tumours have significantly more expression than normal lung tissue, indicating a potential therapeutic marker. CCK-2R siRNAs resulted in up to 97% ( $p < 0.05$ ) knockdown of the receptor in cancer cells, confirming the specificity of LATE-PCR and offering a therapeutic possibility.

The CCK-2R promoter constructs were active in lung, glioma and colorectal cancer cell-lines, demonstrating a potential drug target; however, transcriptional activity did not correlate with gene expression,

suggesting post-transcriptional or translational regulation is a factor affecting CCK-2R expression.

Flow cytometry suggests the presence of a small population of cells within each of these cell-lines which expresses CCK-2R very highly, which was not correlated to CSC markers. However, CCK-2R expression was enriched when cells were grown as spheres, and inhibition caused a delay in sphere-forming, implying that the CCK-2R may play a role in tumour, and CSC, expansion. Thus, CCK2R provides a potential target for therapeutic intervention in cancer.

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# Abbreviations

5FU	5-Fluorouracil
<b>A</b>	
ABCG2	ATP-binding cassette G2
ABI	Applied Biosystems
AC133	Epitope of CD133
Akt	Protein kinase B
ALDH1	Aldehyde dehydrogenase 1
APC	Adenomatous polyposis coli
ARE	AU-rich element
Arg	Arginine
ATF4	Activating transcription factor 4
ATP	Adenosine-5'-triphosphate
<b>B</b>	
Bad	Bcl2-associated agonist of cell death
Bcl2	B-cell lymphoma 2 gene
bp	Base pairs
<b>C</b>	
CagA	Cytotoxin-associated gene product A
CAT	Chloramphenicol acetyltransferase
CCK	Cholecystokinin
CCK-1R	Cholecystokinin 1 receptor
CCK-2R	Cholecystokinin 2 receptor
CCK-2Ri4sv	CCK-2R intron 4-retained variant
CCKR	Cholecystokinin 1 receptor (in Figure 1.13)
CD133	Prominin1
CD24	Signal transducer 24
CD26	A T-cell activation molecule
CD44	A cell surface glycoprotein
CDH1	E-Cadherin (1) gene
CI-988	PD134308; CCK-2R inhibitor
CK20	Cytokeratin 20
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CSC	Cancer stem cell(s)
Ct	Cycle threshold
CTFP	C-terminal flanking peptide
<b>D</b>	
DAG	1,2-diacylglycerol
DNA	Deoxyribonucleic acid
<b>E</b>	
ECF	Epirubicin, cisplatin and 5FU combination therapy
ECL	Enterochromaffin-like
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF-4E	An mRNA cap-binding protein
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
<b>F</b>	
FAP	Familial adenomatous polyposis
Fas	An apoptosis ligand
FcRc	Primer pairs for LATE-PCR
FITC	Fluorescein
FF1/2R	Neuropeptide FF receptor type 1/2
FGF	Fibroblast growth factor
Fos	An early response TF (with JUN)
FS lin	Forward light scatter
<b>G</b>	

G17 or G34	Amidated gastrin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GASR	Gastrin/Cholecystokinin 2 receptor (in Figure 1.13)
gDNA	Genomic DNA
gERE	Gastrin EGF response element
GI	Gastrointestinal
Glioma	Glioblastoma
Gly-gastrin	Glycine-extended gastrin
GnRH-I/II	Gonadotrophin releasing hormone-I/II
GnRH-R	Gonadotrophin releasing hormone receptor
GPCR	G-protein coupled receptor
GRHR	Gonadotropin releasing hormone receptor
GRP	Gastrin releasing peptide
GRP78	78 kDa glucose-regulated protein
GV150013	CCK-2R inhibitor
<b>H</b>	
HB-EGF	Heparin-binding epidermal growth factor
HDGC	Hereditary diffuse gastric carcinoma
HIF-1 $\alpha$ /2 $\alpha$	Hypoxia-inducible factor 1 $\alpha$ /2 $\alpha$
HPNCC	Hereditary non-polyposis colorectal cancer
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HuR	ARE binding protein
<b>I</b>	
IC <sub>50</sub>	Half maximal inhibitory concentration
IHC	Immunohistochemistry
IL-1	Interleukin 1
IL-8	Interleukin 8
IF	Immunofluorescence
IFN- $\lambda$	Interferon-gamma
I $\kappa$ B $\alpha$	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha
IP <sub>3</sub>	Inositol 1,4,5-triphosphate
IRES	Internal ribosome entry site
<b>J</b>	
JAK	Janus kinase
JMV 320	CCK-2R inhibitor
JNK	<i>c-Jun</i> NH <sub>2</sub> terminal kinase
JUN	An early response TF (with Fos)
<b>K</b>	
Kras	Kirsten rat sarcoma viral oncogene homolog
<b>L</b>	
L-365,260	CCK-2R inhibitor
L-740,093	CCK-2R inhibitor
L primer	Limiting primer
LATE-PCR	Linear-after-the-exponential PCR
<b>M</b>	
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
MLH1	<i>mutL</i> homologue 1 gene
MMP	Matrix metalloproteinase
MRP5	Multidrug resistance related protein 5
MSH2 or 6	Mismatch repair homologue 2 or 6 gene
mTOR	Mammalian target of rapamycin
<b>N</b>	
NICE	National Institute for Clinical Excellence
NF- $\kappa$ B	Nuclear factor-kappa B
NPGPR	Alternative splice variant of FF-2R
NPAF	A-18-F-amide
NPFF	F-8-F-amide
NSCLC	Non-small cell lung cancer

<b>O</b>	
Oct4	Octamer-binding transcription factor 4
OX1/2R	Orexin (hypocretin) receptor type 1/2
OXR <sub>s</sub>	Orexin (hypocretin) receptors
OXYR	Oxytocin receptor
<b>P</b>	
P120	Proliferation-associated protein
PCR	Polymerase chain reaction
PEI	Poly(ethylene-imine)
PI3K	Phosphatidylinositol 3 kinase
PIP <sub>2</sub>	Phosphatidylinositol bisphosphate
PLC $\beta$	Phospholipase C $\beta$
PKB	See Akt
PKC	Protein kinase C
PMS2	Mismatch repair endonuclease gene
PMT1 lin	Side light scatter
PPI	Proton pump inhibitor
PTEN	Phosphatase and tensin homolog
PTX	Pertussis toxin
<b>Q</b>	
qRT-PCR	Quantitative real-time PCR
<b>R</b>	
raf	A serine/threonine-protein kinase
ras	Rat sarcoma protein
REG1 $\alpha$	Regenerating islet-derived 1 $\alpha$
(m)RNA	(messenger) Ribonucleic acid
RNAi	RNA interference
<b>S</b>	
SCLC	Small cell lung cancer
SCM	Stem cell medium
Shc	Src homology domain containing protein
siRNA	Short interfering RNA
SP	Signal peptide
SP1	Specificity protein 1
Sox-2	SRY-box 2
Src	Sarcoma-inducing tyrosine kinase
SRY	Sex determining region Y
SSTR2	Somatostatin receptor 2
<b>T</b>	
TCF-4	Transcription factor 4
TF	Transcription factor
T <sub>m</sub>	Melting temperature
TNF- $\alpha$	Tumour necrosis factor-alpha
<b>U</b>	
U	Undetectable (in qRT-PCR)
U2AF35	A splicing factor
<b>V</b>	
V1A/1B/2R	Vasopressin receptor type 1A/1B/2
VR <sub>s</sub>	Vasopressin receptors
VC	Vector control
VEGF	Vascular endothelial growth factor
<b>W</b>	
wt	Wild-type
<b>X</b>	
X primer	Excess primer
XIAP	X-linked inhibitor of apoptosis
<b>Y</b>	
YM022	CCK-2R inhibitor
<b>Z</b>	

Z360  
ZBP89

CCK-2R inhibitor  
Zinc-binding protein 89



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# Chapter 1 Introduction

## **1.1. Cancer**

Cancer is a cellular disease with many different causes. The cells involved become deregulated and do not respond to normal growth limitations, allowing a mass of cells, or a tumour, to develop in a process called carcinogenesis. 5-10% of cancers are caused by an inherited genetic fault, whereas environmental factors, such as infection or exposure to radiation, are the cause of the DNA damage in the rest of cases [1]. Genes become mutated, allowing loss of tumour suppressor genes; which stop excess growth in the normal situation, or gain of oncogenes; those genes which promote expansion and replication. The cells lose normal function and can begin to spread to other tissues, causing damage throughout the body. This is called metastasis and along with invasion of the lymph nodes is a late stage in cancer and usually associated with a poor prognosis of survival. This, however, varies between cancers, as do treatment options, drug effectiveness and initial risk factors.

Cancers are different with regard to cell type, position, blood vessel recruitment and genotype, but they all display certain hallmarks. These were originally six physical properties of cancer cells themselves [2], but have evolved into 12 hallmarks, including those of the environment in which the tumour grows, for example oxidative stress (Figure 1.1) [3].

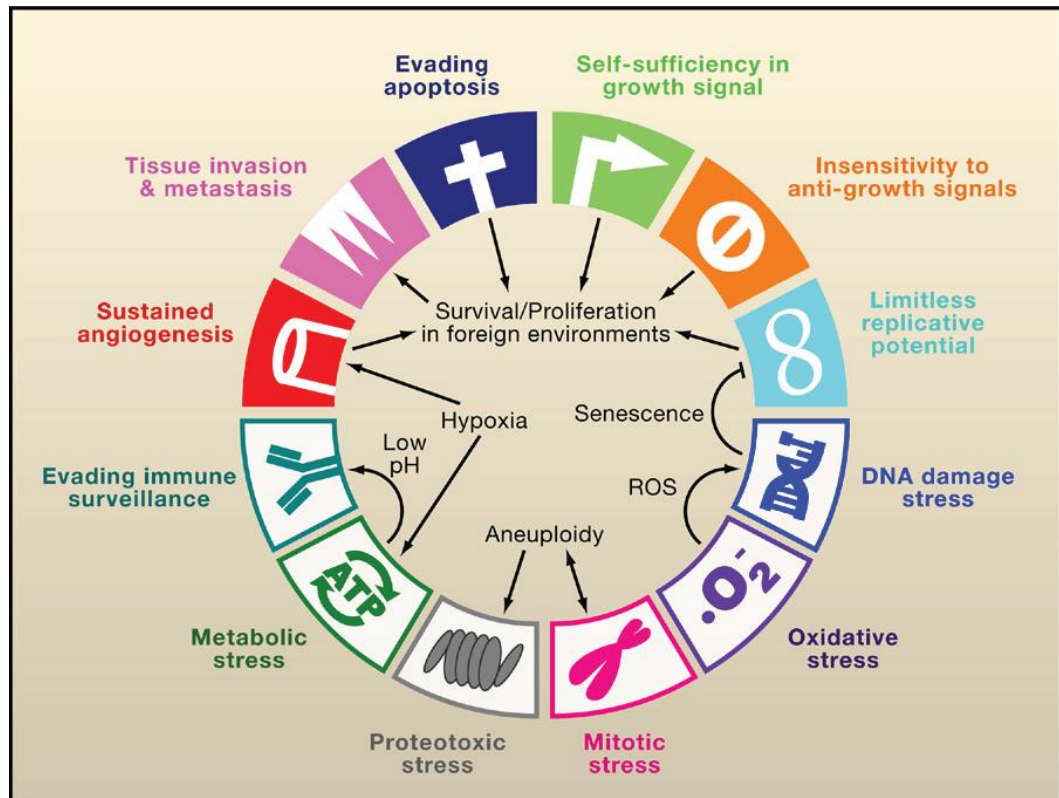


Figure 1.1: The hallmarks of cancer [3]

The cancers of relevance to this project include gastrointestinal (GI) forms: gastric adenocarcinoma, colorectal carcinoma, oesophageal carcinoma and pancreatic carcinoma. GI cancers are clinically important due to their late progression and consequent poor prognosis. They are all solid cancers of the epithelium. Also of particular interest are lung carcinomas, especially small cell lung cancer (SCLC), and glioblastoma brain tumours.

#### 1.1.1. Gastric adenocarcinoma

Gastric adenocarcinoma, is the second most common cause of cancer death in the world [4, 5], with over 900,000 cases diagnosed annually worldwide. It is a lingering disease, normally caused by long-term inflammation of the gastric epithelium. This inflammation has multiple causes such as regular use of anti-inflammatory drugs, tobacco and alcohol, or consuming large quantities of pickled foods [4], but the most



common is known to be *Helicobacter pylori* infection [5]. Once the stomach lining has become inflamed, the cells start to develop mutations in their DNA, causing atrophic gastritis, a premalignant condition. The epithelial cells progress through intestinal metaplasia and dysplasia until eventually gastric adenocarcinoma is diagnosed [6].

*H. pylori* can cause up-regulation of various cellular factors including pro-angiogenic proteins, and so this pathogen is a potent carcinogenic risk [7]. This conclusion is supported, cautiously, by Danesh [8] in their 1999 review of the many trials carried out. But in 2004 Correa [9], stated that infection with *H. pylori* is the 'driving force' in gastric carcinogenesis, and that eradicating the bacteria from certain patients led to no cases of the cancer in those people. Certain strains of *H. pylori* are considered to be more of a risk, such as cytotoxin-associated gene product A (CagA)-positive bacteria. CagA-strains are associated with greater virulence and far more cases of gastric cancer than CagA-negative strains [6].

Gastric cancer can also be hereditary, with the best known of these arising from the mutated E-cadherin gene, CDH1, a tumour suppressor gene. This produces hereditary diffuse gastric carcinoma (HDGC) which often presents in younger patients (<age 45) with a strong family history of the condition [10]. This gene is screened for in those families thought to be at risk, so that prophylaxis can be an option.

The treatment for gastric cancer generally starts with major surgery to eliminate the tumour, and in some cases this will mean removing the entire stomach [4]. Chemotherapy can reduce tumour size before the operation or it can be used as palliative care, to keep the carcinoma from growing. The most effective combination therapy for gastric cancer is ECF, which includes the drugs Epirubicin, Cisplatin and 5-

Fluorouracil (5FU) [4]. However, other drugs can be used if these fail to produce a decrease in tumour size, and radiotherapy can be undertaken if these are unsuccessful. The latter is not a normal treatment for gastric carcinoma though, and is usually used only as palliative care.

#### 1.1.2. Oesophageal carcinoma

In direct opposition to the above, it has been found that infection with *H. pylori*, and the cytokines expressed, may protect the oesophageal mucosa from developing adenocarcinoma [9]. Although there are half as many diagnoses of this cancer (400,000 worldwide per annum) compared to that of gastric carcinoma, the 5-year survival rate is lower at 8% as opposed to 15% [11, 12]. This is due to the fact that metastasis has often already taken place by the time of diagnosis, allowing for fewer treatment strategies. Again this disease is a consequence of chronic inflammation of epithelial cells, this time always caused by repeated acid reflux from the stomach into the oesophagus. Alcohol consumption, smoking tobacco and being excessively overweight are also powerful risk factors, as they are for most cancers [11, 12]. Hormones too, can play an important part in causing Barrett's oesophagus, a premalignant condition, to progress to adenocarcinoma, by impairing the cell's apoptosis pathways [13]. This cancer is associated with a mutation in p53, a critical tumour suppressor gene, involved in cell cycle control, DNA repair and instigating apoptosis.

Treatment for oesophageal carcinoma normally involves removal of most of the oesophagus, causing these patients major problems with nutrition [11]. Once the tumour is removed a part of the stomach or colon is positioned in its place in order to allow digestion but this is not ideal. If the cancer cannot be removed a stent is put inside the oesophagus in order to improve a patient's quality of life, and

chemotherapy, radiotherapy or a combination of both are used to reduce tumour size [11]. In some cases this can completely cure the cancer, but in others it will be used for palliative purposes.

### 1.1.3. Colorectal carcinoma

Colorectal carcinoma was diagnosed in over one million people across the world in 2002 [14]. However, the five-year survival rate is much higher than the cancers above, at around 51% [14]. This is likely to be due to the fact that this is one of the few cancers for which screening is possible. Risk groups (those aged 60-69 in England [15], or 50-74 in Scotland [16]) are screened for faecal blood and if this is found then a colonoscopy is performed. This procedure examines the bowel lining for polyps or other anomalies, and these can then be treated, sometimes before the adenocarcinoma develops. This cancer is often caused by environmental factors, the most common being excessive red meat and fat, and lack of fibre in the diet [17]. There are also two hereditary causes of colorectal cancer, accounting for 5% of all cases. These are a mutated adenomatous polyposis coli (APC) gene, causing familial adenomatous polyposis (FAP) [18], or a mutated variant of one of a set of DNA repair genes, MSH2, MSH6, MLH1 and PMS2, which bring about hereditary non-polyposis colorectal cancer (HNPCC) [19].

In the normal colon APC binds and down-regulates  $\beta$ -catenin, a role which regulates the transcription of many genes including those involved in control of the cell cycle and migration. Mutation (point or gross deletion) of the APC gene in FAP leads to the introduction of a premature stop codon and therefore truncation of the APC protein in 94% of cases [20]. This protein cannot bind to  $\beta$ -catenin causing constitutive activation of the Wnt pathway, and more transcription of growth promoting genes, allowing adenomas to develop in the colon [20]. The mutation site is important since it predicts the severity of the

disease; if it falls within codons 1250-1464 then the patient will develop far more adenomas than if the mutation is at the very ends of the gene [18]. The number of adenomas ranges from 5000 in the first group to 100 in the second, and this in turn correlates with the likelihood and age at which colorectal carcinoma develops; the more adenomas the worse the prognosis [18].

Most patients with colorectal cancer are subjected to some kind of surgery to remove their tumour [14]. This treatment is successful if the cancer is in its early stages, and often the bowel section is removed and the ends are joined up to cause little digestive disruption. However, if the carcinoma is advanced a large portion of the colon can be taken out and a colostomy bag will replace it. Chemotherapy can be used to reduce colorectal tumour size and the most commonly used drug is 5FU [14]. Irinotecan, cisplatin and oxaliplatin can also be used for more advanced carcinoma cases. Radiotherapy is not often used, except in palliative cases [14].

#### 1.1.4. Pancreatic carcinoma

Over 200,000 people worldwide were diagnosed with pancreatic cancer in 2002 [21, 22], and due to the late appearance of symptoms this disease has an appalling five-year survival rate of 2-3% [21, 23]. It is associated with smoking and is most often seen in the elderly [22, 23]. The three cancers above can be at least partially treated with surgery to remove tumour growth. However, with pancreatic carcinoma this is not very successful, due to the late stage of most diagnoses and the complexity of the surrounding tissues [24]. An almost universal mutation in pancreatic carcinoma is that of Kras, an oncogene capable of activating many pathways in carcinogenesis [25].

Radiotherapy or chemotherapy or a combination of the two can be used to shrink the tumour either to prepare for surgery or to relieve pain and discomfort [26]. Chemotherapy is the first line of treatment for advanced cancer, with the drug, gemcitabine, being recommended by the National Institute for Clinical Excellence (NICE). However, this is only undertaken if the patient is well enough to cope with the side-effects [26]. It was hoped that perhaps hormonal therapy, such as blocking some growth-enhancing receptors, may have worked to impede tumour expansion. Many studies of the trophic effects of gastrointestinal hormones have been carried out, but have come up with no clear conclusion. Some studies seem to show that these hormones could enhance tumour growth and carcinogenesis, and others the opposite effect [24].

#### 1.1.5. Lung carcinoma

Lung cancer is the most common form of cancer diagnosed throughout the world today, with 1.3 million new cases identified in 2002 [27]. Unfortunately it is also the most common cause of cancer death, with between 7 and 9% of patients surviving 5 years after diagnosis [27]. The disease is largely restricted to the elderly, and is most frequent in smokers; however, passive smoking, exposure to industrial carcinogens and air pollution all contribute to the risk of developing lung cancer.

SCLC makes up between 20 and 25% of lung cancer cases, and despite being highly responsive to chemotherapy; most patients relapse and die from chemo-resistant disease [28]. The accepted regimen for early-stage SCLC is cisplatin plus etoposide, and this is effective short-term in 85% of cases [27, 28]. Radiation therapy is considered as palliative care for those with late-stage disease or recurrent tumours. Non-SCLC (NSCLC) has a better diagnosis, with patients detected at an early stage being eligible for major surgery, allowing a 60% 5-year

survival rate [27]. Surgery is the first resort for patients, and then chemotherapy or radiotherapy can be used instead of surgery. In palliative cases chemotherapy and relief of symptoms such as lung obstructions are combined.

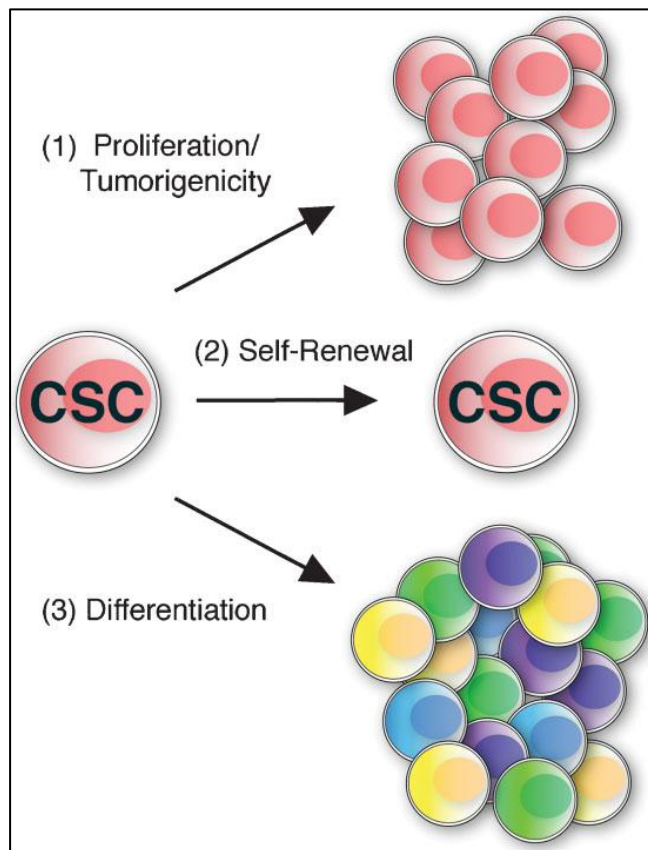
#### 1.1.6. Glioblastoma

Brain tumours make up around 2% of all cancers in the UK and there were over 4500 diagnoses in 2006 [29]. Glioblastomas (gliomas) make up about 50% of these cases and often develop from astrocyte cells in the brain [30]. Survival rates for high and low grade gliomas range from a few months to a few years [31].

Focal gliomas tend to develop in children, meaning that the border of the tumour is easily defined allowing for more successful surgery. Unfortunately adult gliomas are more diffuse, and can form in very difficult areas such as the brain stem. In this case neither surgery or radiotherapy are treatment options and survival will be very limited [30]. However, in certain situations of age, location and growth rate surgery, chemotherapy and radiotherapy are all options for treatment of glioblastoma [30].

### 1.2. **Cancer stem cells**

Cancer stem cells (CSC) are postulated to exist within many, if not all, cancers [32-34]. CSC are the cells which initiate and maintain the tumour, which makes them especially relevant in the case of chemotherapy, where they can lie dormant and allow recurrence. There are three defining characteristics of CSC identified by Schatton *et al.* and these are a) the capacity to produce tumours, b) identical regeneration and c) to produce non-stem cancer cells (Figure 1.2) [35].

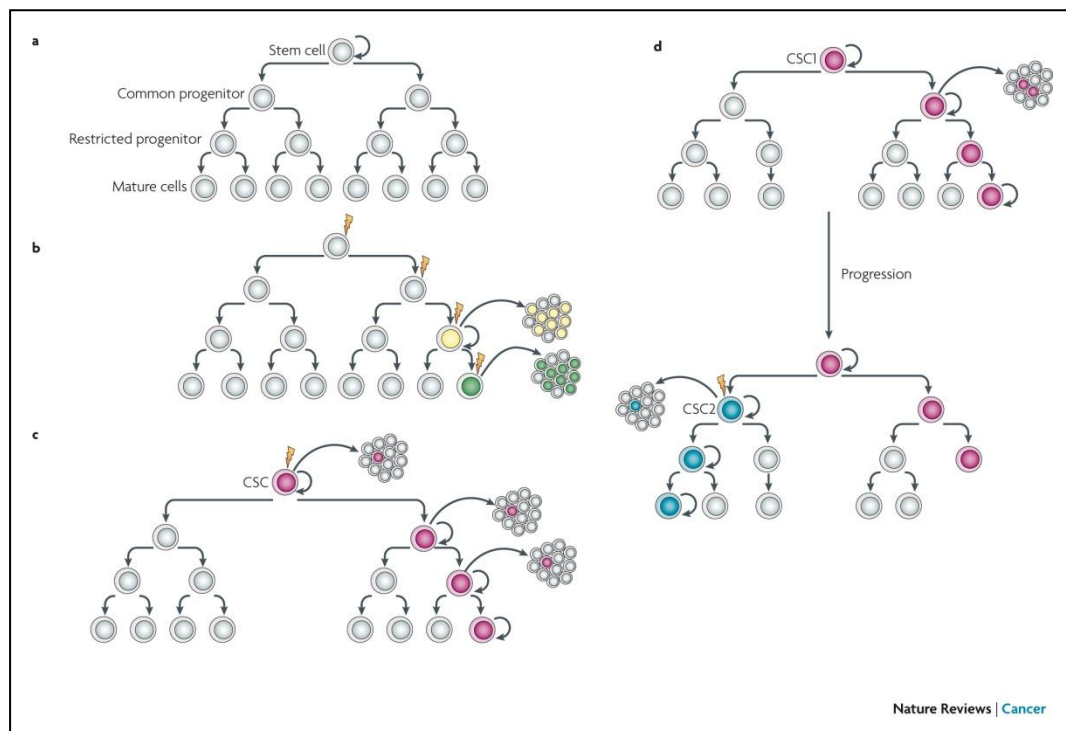


**Figure 1.2:** In order to be classed as such, cancer stem cells must show the ability to proliferate into a tumour, self-renew and produce differentiated cells [35]

CSC were first identified when it was found that only a subset of leukemic cells taken from an acute myeloid leukaemia patient could regenerate the disease in immunocompromised mice [36]. This challenged the old stochastic model of tumour initiation; where any cell in the tumour could propagate the cancer [37]. A review by Schatton *et al.* indicates that CSC have been found in breast, brain and ovarian cancer and melanoma, and more recently in colon carcinoma [34, 35]. However, the membrane markers that distinguish the CSC in each cancer type are different. For example the markers of stemness in leukaemia are those that mark normal stem cells; CD24 and CD44 [38]. The main cancer stem cell marker known in brain cells is CD133; however this molecule is not sufficient in colorectal cancer cells to indicate a cancer-initiating cell.

### 1.2.1. Cancer stem cells in tumour formation and growth

One of the major problems with identifying CSC markers is that in some cases CSC can generate a xenograft tumour from just 20 cells [39, 40]. The CSC model of tumour growth asserts that the actual number of cells needed to form a tumour is just one; however, the nature of xenograft formation does not make this simple to demonstrate (Figure 1.3). This means that the probability of contamination with cells of a different phenotype is very high, however the CSC are isolated [39].



**Figure 1.3: Models of tumour propagation**

*There are several theories about tumour initiating cells. a) shows the normal tissue situation, where stem cells give rise to differentiated cells, which form an organ. b) is the stochastic model or clonal evolution; where all cells have the same tumourigenic capacity. c) the CSC model; one cell gives rise to the tumour cell bulk while renewing itself. d) shows the clonal evolution of the CSC into a second distinct cell (CSC2) which becomes dominant due to further mutations and goes on to propagate the tumour.[39]*

Another debate which continues is whether the CSC arises from a mutated stem cell or a progenitor cell which has mutated to become stem-like, i.e. demonstrates self-renewal [41, 42]. There are even suggestions that a somatic cell merges with a stem cell in some cancers to form the CSC [41]. Unfortunately this question will remain



unanswered until a definitive population of CSC can be found within any given tumour.

### 1.2.2. Cancer stem cell markers

The only way to diagnose the tumour initiating cell for certain is to test its ability to produce the same tumour in an immunodeficient mouse [43]. *In vitro* assays such as sphere-forming have also been used to select for the CSC population, since these 3D spheres are more similar to the tumour environment than 2D culture [44, 45]. However, there is some controversy over exactly which markers illustrate CSC. CD133 was the first marker linked to CSC in neural cancer cells [46], but in colorectal carcinoma cells different groups have found that CD133<sup>-</sup> cells cannot [47] and can [48] produce tumours in mice. The first study states that since only CD133<sup>+</sup> cells can produce tumours, these must be the tumour-initiating population and therefore the CSC [47]. The second study overturns this research, showing widespread CD133 expression in the primary tumour colonic epithelium, and demonstrating that the CD133<sup>-</sup> cells are in fact the more tumourigenic population [48].

There have been many markers linked to colon CSC. These include survivin and multidrug resistance related protein 5 (MRP5), which were shown to be related to faster progression of colon cancer by Gazzaniga *et al.* [49]. They also showed that aldehyde dehydrogenase 1 (ALDH1) was associated with poor prognosis but that CD44 and CD133 expression were not, in colorectal cancer patients. Conversely, Lugli *et al.* suggest that loss of CD166 and CD44s may lead to a worse prognosis [43].

Another study by Pang *et al.* showed that CD26 was up-regulated in metastasis compared to unpaired primary tumour samples [50], which they relate to CSC status. CD26<sup>+</sup> tumour cells were capable of liver

metastasis in a mouse model, with or without CD133/CD44 expression, but expression of these 2 markers did increase tumourigenicity. CD26<sup>-</sup> cells could not metastasise independent of CD133/CD44 status [50]. From this data they hypothesise that two types of CSC exist; a stationary one promoting tumour growth and a metastatic CSC.

Drug-resistant tumour cells display up-regulation of drug transporters, for example ABCG2, which allows the cell to pump out any chemotherapy used in the treatment of cancer patients [51]. This has led to the hypothesis that ABCG2 is a CSC marker, since CSC are the cells from which the tumour recurs after chemotherapy has been applied and therefore must be resistant to such therapy. ALDH1 has also been put forward as a marker since this would enhance CSC survival by detoxification of cellular aldehydes [52].

Very recently Vermeulen *et al.* have hypothesised that the HCT116 (colorectal carcinoma) cell-line does not contain a CSC fraction as they are not hierarchical in organisation [53]. They implicate Wnt in a hierarchical organisation, showing that Wnt activity is correlated with many possible CSC markers, CD133, CD166, CD24, CD29 and CD44. This hypothesis is supported by various experiments in which they grew tumours from cells displaying high Wnt activity. They assert that their previous data illustrated that colon CSC lose their markers when grown in medium with serum, therefore stroma must be a part of the CSC niche, and more particularly the myofibroblasts. When myofibroblasts were mixed with low Wnt activity cells (which are differentiated), they induced several cells to revert to the CSC phenotype of high Wnt activity [53].

Another Wnt target gene, Lgr5, has been associated with CSC since it is a stem cell marker in colon cells [54]; the Lgr5<sup>+</sup> cells at the base of

each colon crypt undergo rapid cycling, and appear to give rise to other cells in the crypt. Vermeulen *et al.* found that Lgr5 expression correlated with their high Wnt activity CSC [53]. Lgr5 is also implicated in the metastasis of colorectal carcinoma cells, although the authors admit more research should be done to prove this connection [37].

In lung cancer, the consensus so far is that stem cells can be identified by CD133 and ABCG2 expression [55]; with the latter allowing efflux of Hoechst 33342 dye, allowing the cells to be seen as a side population using flow cytometry [56]. Increased Wnt signalling is also seen in the lung CSC subset, and this can be confirmed by build up of  $\beta$ -catenin in the nuclei of the cancer cells [57].

Glioblastoma CSC markers are less controversial than those in either colorectal or lung cancers. CD133-positive cells alone can initiate tumours [46] and are more highly associated with the most aggressive tumours [58]. These neural CSC have been linked to stress conditions, for example hypoxia allows for the maintenance of the CSC subset, through the activation of HIF2 $\alpha$  [55]. This molecule in turn up-regulates *Oct4*, which is a normal stem cell marker, recently validated as a CSC marker [59].

It is likely that CSC can be heterogeneous, which explains why certain markers have been proved and disproved in the same cancer type; e.g. CD133 in colorectal carcinoma [39, 47, 48, 53]. In the future cancer types may be separated due to their CSC subtype.

### **1.3. Hormones in cancer**

The focus of this project is to find out how cancer is affected by the hormone, gastrin, via its receptor, the cholecystokinin 2 receptor (CCK-

2R). Cancers as a group have many diverse direct causes and risk factors. The progression of cells from normal to cancerous; carcinogenesis, consists of disruption to cell proliferation, angiogenesis, cell adhesion and cell death or apoptosis. Since these processes are under hormonal control in the body, hormones can initiate, promote or be affected by cancer [60]. A potent initiator and promoter of carcinogenesis is oestrogen. This hormone can, in some cases, induce changes in cellular DNA and in this way can initiate endometrial cancer [61]. In its role as a promoter oestrogen can also augment cell division and proliferation, causing tumours to develop, often in reproductive tissues.

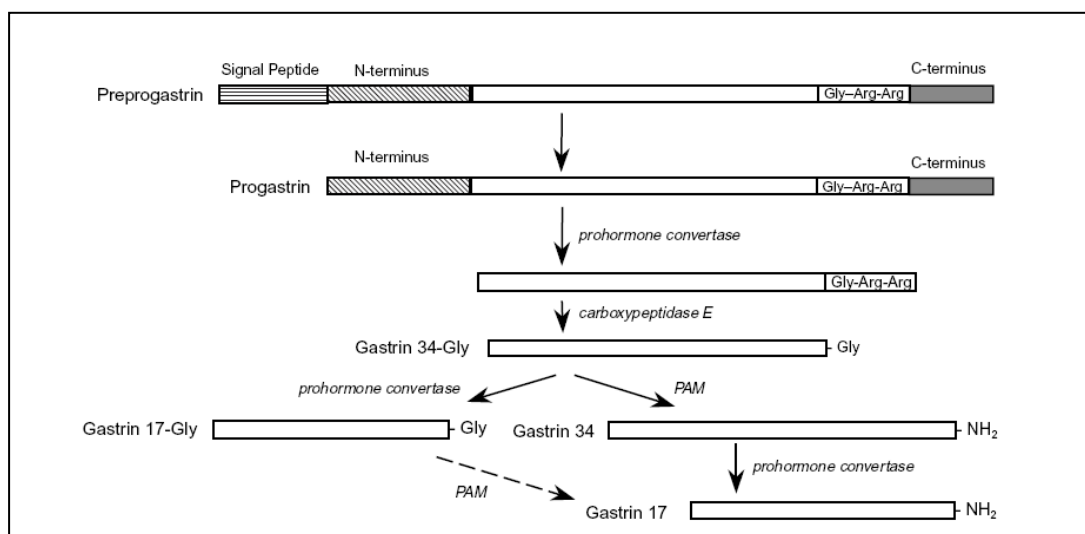
The gastrointestinal cancers described above, as well as being initiated and promoted by hormones, also affect certain hormone expression. This in turn can lead to further promotion by the hormone which has been up-regulated, leading to a feedback mechanism. However, in modern medicine this link can be exploited, since these cancers are often responsive to hormone therapy, such as the use of hormone receptor antagonists [62]. Many GI hormones have been linked to carcinogenesis, for example cholecystokinin (CCK) has been associated with increased proliferation of pancreatic tumour tissues and also in facilitating invasion across basement membranes [60, 62, 63].

Another GI hormone, gastrin, is a well-known promoter of all the GI cancers and although not usually expressed in the normal tissues, is produced in these and many other cancer cells [62]. Gastrin releasing peptide (GRP), its analogue; bombesin, and neurotensin have been found to have promoter activity in gastric carcinoma, although the latter was only studied in rats [60]. Neurotensin has been shown to stimulate human and murine colorectal cancer, though, and this seems to be due to its stimulation by certain fats [60]. This project will focus on the

effects of gastrin on GI and lung cancer and glioblastoma, and the receptor which mediates its effects.

#### 1.4. Gastrin

Gastrin is the hormone which causes gastric acid to be secreted into the stomach. It is also able to cause epithelial proliferation leading to growth of the gastrointestinal mucosa [64], and eventually to stomach ulcers. In addition it can bring about enterochromaffin-like (ECL) and parietal cell maturation [65]. The translated product of the gastrin gene is preprogastrin, a 101-amino acid peptide. This is cleaved of its 21-amino acid N-terminal signal peptide (SP) in the ER/Golgi apparatus, to become progastrin (Figure 1.4). Further processing in endocrine cells cleaves this peptide at Arg94-Arg95 to form glycine-extended gastrin (Gly-gastrin) and eventually amidated gastrin (G17 or G34) [66, 67].



**Figure 1.4: Gastrin biosynthesis showing each of the intermediate, and biologically active, peptides**

*(The dashed arrow has only been shown by certain authors (see [66] for more information.) [66]*

Gastrin is produced in the G cells of the antro-pyloric mucosa, where the enzymes necessary for processing (Figure 1.4) are expressed. These include signal peptidase, to remove the SP, prohormone convertase, which cleaves progastrin and peptidyl  $\alpha$ -amidating

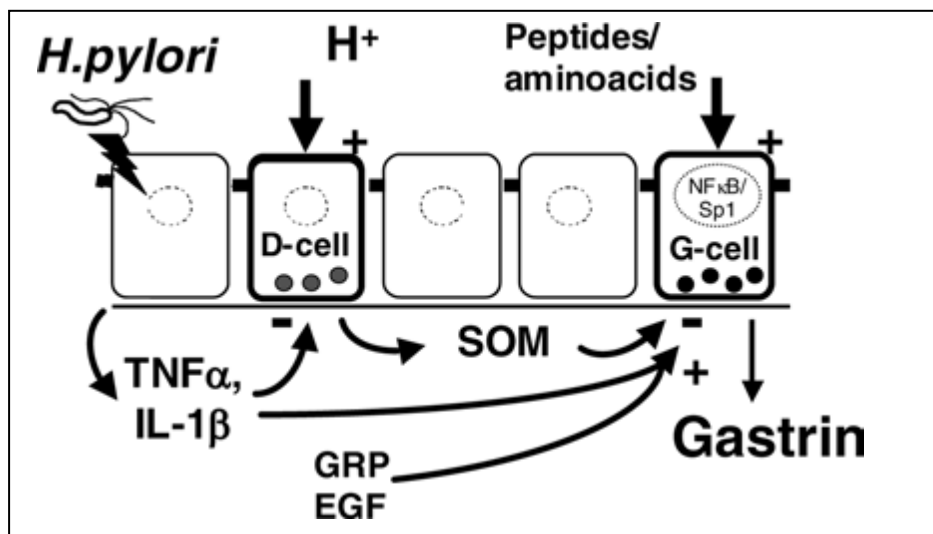
monooxygenase to produce amidated gastrin [66, 67]. This is then exocytosed from secretory granules to be transported to the oxyntic mucosa [68]. Here it can interact with the CCK-2R on ECL cells. The stimulated ECL cells release histamine, inducing the parietal cells to produce and discharge gastric acid into the stomach lumen [69]. The stomach is therefore kept in a constant acidic state (pH 1-2) in order that various digestive enzymes can operate. Gastrin may also be able to directly stimulate parietal cells [70].

#### 1.4.1. Gastrin in cancer tissues

However, gastrin is able to influence much more than just digestion [64]. It is transcribed in a lot of different pre-malignant and tumour cells. 48% of gastric cancers are gastrin gene positive, along with 80-90% of colorectal polyps, 78% of pancreatic tumours [71] and 100% of oesophageal cancers [72]. Gastrin expression is also found in many non-GI cancers such as ovarian tumours and bronchogenic carcinomas. It is present in pre-malignant cells as well as full-blown cancer and is proved to be a promoter of the carcinogenic process when combined with a co-factor. Examples of co-factors are bacterial infection (e.g. *H. pylori*), inflammatory cytokines (tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1)) and the presence of mutant cells. Gastrin may also influence stem cells via regenerating islet-derived 1 $\alpha$  (REG1 $\alpha$ ) [71]. It has been shown that increasing levels of gastrin correlate with rising severity of illness and a greater degree of metastasis [73], especially in pancreatic and colorectal cancer cells.

Gastrin expression is regulated by a number of factors, some acting before transcription and others post-translation. Gastrin transcription can be significantly increased by infection with *H. pylori* [74] due to various proteins secreted by the bacterium itself (Figure 1.5). Gastrin transcription is also induced by epidermal growth factor receptor

(EGFR) ligands (e.g. EGF) via the EGF response element on the gastrin promoter (gERE) [75]. EGF can increase expression of the gastrin gene through a transcription factor (TF), SP1, and this introduces the possibility that gastrin may be regulated by ras [75]. Another molecule, JUN, mediates this interaction, and is in turn negatively mediated by somatostatin, the hormone which causes inhibition of gastrin expression. Another transcriptional alteration can be achieved through the zinc-finger TF (ZBP89) binding to the gastrin promoter causing repression [76].



**Figure 1.5: Regulation of gastrin release from the G cell**

*Peptides and amino acids are responsible for normal digestive release, however the other factors shown are either disease-led (*H. pylori* and its cytokines) or can be altered in cancer (GRP, EGF) [77]. SOM: Somatostatin, GRP: Gastrin releasing peptide, EGF: Epidermal growth factor.*

Post-translational up-regulation of gastrin occurs by altering secretion from G cells. This can be caused by administration of proton pump inhibitor (PPI) drugs [78]. PPI drugs are prescribed for the treatment of excess stomach acid and/or reflux and can bring about sustained hypochlorhydria (low stomach acid), which in turn can lead to compensatory hypergastrinaemia [79]. Another factor is the bacterium, *H. pylori*, which in addition to its direct effects, causes infected cells to produce high levels of cytokines (Figure 1.5). These include interferon-gamma ( $IFN-\lambda$ ),  $TNF-\alpha$  and interleukin-8 (IL-8) [80] and can increase

proliferation of G cells and their secretion of gastrin into the stomach epithelium [80].

A third post-translational up-regulator of gastrin is GRP, although this actually has a negative effect on gastrin expression in the fundic mucosa [60]. Somatostatin is a negative regulator of gastrin up-regulation and its release from G cells, and so can cause a large drop in levels of the hormone. The cytokines associated with *H. pylori* infection also cause a drop in D cell production of somatostatin (Figure 1.5), therefore increasing gastrin by removing this inhibition [80]. All three of these, gastrin, GRP and somatostatin, bind to G-protein coupled receptors (GPCRs) in order to affect a cell [71, 81].

#### 1.4.2. Gastrin peptides in carcinogenesis

When gastrin production takes place in non-endocrine cells, the process becomes less efficient at assembling amidated gastrin; the usual end result, due to the necessary enzymes being available only at limiting concentrations [77]. The consequence of this is that molecules of Gly-gastrin and progastrin; the normal intermediates (Figure 1.4), accumulate in the tissues [71]. This tends to occur in cancerous tissues, such as colorectal carcinomas, where immature forms of gastrin may overshadow its mature forms.

Gly-gastrin, progastrin and the C-terminal flanking peptide (CTFP; cleaved from progastrin; dark grey portion in Figure 1.4) have been shown to be biologically active. They are linked to both the digestive function of amidated gastrin, maintaining acid secretion, and the carcinogenic function, accelerating tumour development [67]. Both progastrin and Gly-gastrin have been shown to enhance growth of many different cell-lines *in vitro* and *in vivo* and to up-regulate anti-



apoptotic proteins [82]. However, these products do not have so large an effect on gastric secretion as amidated gastrin.

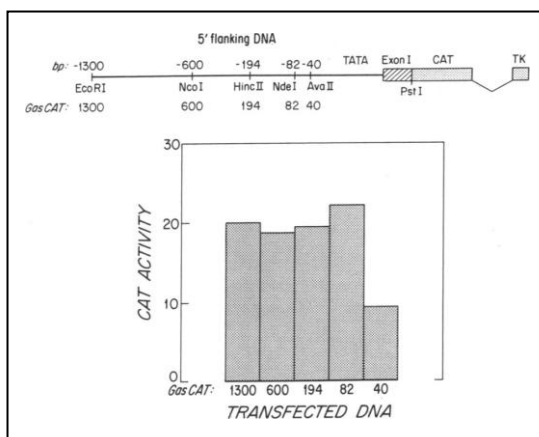
Gly-gastrin was the first of these intermediates to be studied. Seva & colleagues [83] investigated the effect of gastrin and Gly-gastrin on AR42J cells (a rat exocrine pancreatic cell-line) and found that they could both stimulate cell proliferation to a similar degree. Since this discovery, proliferative activity has also been shown for progastrin and the CTFP [66]. Gly-gastrin is found in large quantities in human colon and lung carcinoma cells, and when over-expressed in mouse models it can cause intestinal polyposis and bronchoalveolar carcinomas [84]. It has also been found to increase metastasis of LoVo human colon cancer cells [85]. Mürköster *et al.* [86] agree that Gly-gastrin can stimulate colon carcinoma cell proliferation, but that gastrin alone is actually instrumental in inducing apoptosis in these cells. These claims would indicate a large role for Gly-gastrin in colorectal cancer, since the carcinogenic effects must be being accomplished by this peptide alone, and even against the effects of gastrin itself.

Progastrin too has a strong link with colorectal cancer. In 2001, Siddheshwar *et al.* [87] found that progastrin levels were much higher in patients with colorectal cancer (26.8% had progastrin <15 pmol/l), than either those with polyps (58.6%) or the control group (92.5%). However, in the same groups, Gly-gastrin and amidated gastrin concentrations did not fluctuate significantly. Although amidated gastrin levels were higher in those patients infected with *H. pylori* in all three groups, the pathogen did not increase progastrin concentrations. Progastrin has now been found to have an anti-apoptotic effect on colon and pancreatic cancer cells, as well as provoking growth in these cells [88].

The CTFP has been recently studied by Smith *et al.* and found to be able to promote growth in gastric and colon cancer cell-lines as well as encouraging invasion by the gastric cells [67]. It would also seem that concentrations of the CTFP in the circulation of healthy humans and cancer patients are far higher than that of progastrin or Gly-gastrin making this activity all the more significant [67].

### 1.5. Regulation of gastrin expression

The 1300bp upstream of the human gastrin gene has been reported as the promoter of gastrin gene expression by several groups [89, 90]. Godley & Brand [89] produced several plasmid reporter constructs containing between -1300 and -40bp of this upstream DNA, plus the first (non-coding) exon of the gastrin gene, attached to a chloramphenicol acetyltransferase (CAT) gene (Figure 1.6). All of the constructs show promoter activity, but the smallest (40 GASCAT) shows only 50% of that of all the rest (Figure 1.6) [89].



**Figure 1.6: Gastrin promoter constructs**

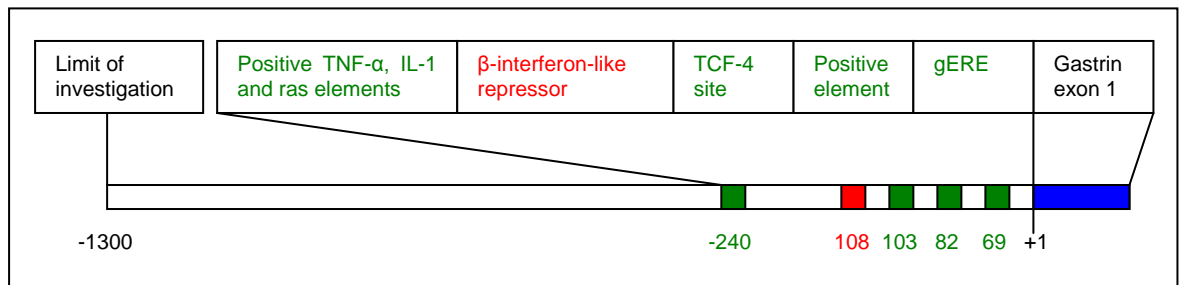
The top diagram shows how a series of gastrin promoter reporter constructs (1300, 600, 194, 82 and 40 GASCAT) were made by Godley & Brand. The graph shows the results of their transient transfection into GH<sub>4</sub> cells, in order to test for promoter activity using CAT [89].

This result is supported by Merchant *et al.* [90] who showed that a construct containing the entire gastrin gene, plus 1300bp upstream and 2500bp downstream of the gene, caused expression of gastrin when stably transfected into GH<sub>4</sub> cells [90]. They also used constructs containing 82bp or 40bp of upstream DNA plus exon 1 and found that the latter had less activity. Further study of this section of DNA caused Wang & Brand to believe that a positive element lies between 82-79bp from the gastrin gene [91] (Figure 1.7). This report also found that

promoter activity in islet cells increased when the construct was shortened to only 82bp of upstream DNA. Again further investigation of the DNA between 194-82bp revealed an 8bp repressor element which matches that found in the  $\beta$ -interferon promoter, at approximately 108bp from the gastrin gene [91] (Figure 1.7).

The gastrin promoter is up-regulated by several factors, including EGF [89, 90], TNF- $\alpha$  [92], IL-1 [93] and ras [94]. A gERE is located between -69 and -40bp upstream of the gastrin gene (Figure 1.7), since removal of this section of DNA from the construct knocked out EGF induction of gastrin expression [92]. It was also found that TNF- $\alpha$  and Fos could stimulate gastrin expression via the promoter [92]. This work was supported and extended by Suzuki *et al.* who showed that TNF- $\alpha$  and IL-1, both present in *H. pylori* infection, could stimulate gastrin expression in the gastric cancer cell-line, AGS [93]. The element(s) involved in the mechanism are unknown, but exist within 240bp of the gastrin promoter, and are not related to the gERE discussed above (Figure 1.7) [93]. In colon cancer cells the oncogene product, ras, has been implicated as an inducer of gastrin expression, again via the first 200bp section of the DNA upstream of the gastrin gene [94].

Koh & colleagues found a transcription factor-4 (TCF-4) binding site between -103 and -93bp on the gastrin promoter (Figure 1.7), allowing gastrin to be up-regulated by the  $\beta$ -catenin/TCF-4 signalling pathway, which has been linked to intestinal proliferation [95]. This can be a cyclical pathway, since gastrin also causes the stabilisation of  $\beta$ -catenin, and therefore enhances transcription of other  $\beta$ -catenin target genes [96].



**Figure 1.7: Diagram representing the putative gastrin promoter**

*1300bp of the putative gastrin promoter has been investigated for positive and negative (shown as green and red boxes respectively) regulatory elements. Exon 1 of the gastrin gene is shown as a blue box.*

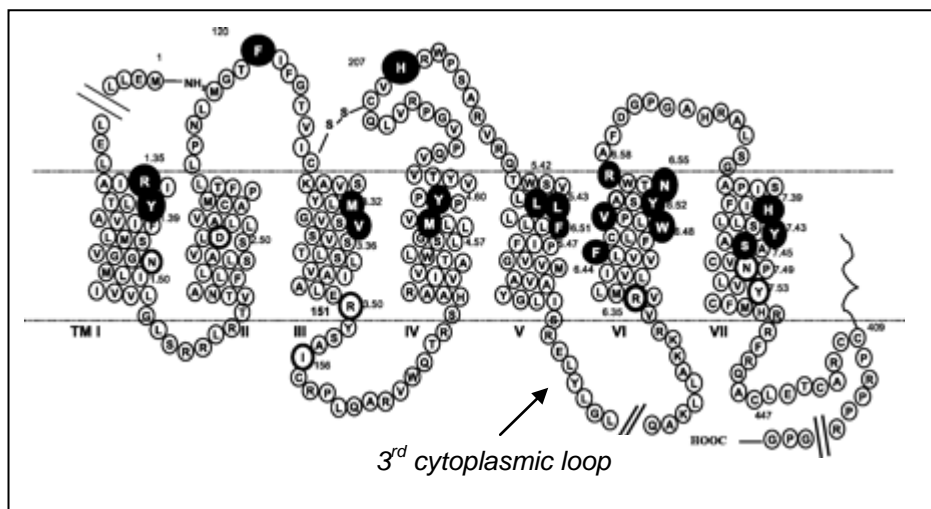
Although 1300bp of the putative gastrin promoter have been investigated so far, it seems to only be the first 200bp section which contains any regulatory elements. However, this may be due to the fact that the literature has focussed on those portions which cause a visible effect in reporter studies. There are many other transcription factor binding sites such as SP1 [75] in the promoter, along with more TCF-4 binding sites, to be investigated in further studies [95].

Additionally an internal ribosome entry site (IRES) has been found within the gastrin transcript in GI cancer cells, suggesting it may be translationally as well as transcriptionally regulated [97]. The IRES allows translation to continue through periods of cellular stress, meaning that angiogenesis can be maintained and apoptosis halted in these cells [97].

## 1.6. CCK-2R

There are two CCK receptors; CCK-1R and CCK-2R [98], and they share 50% amino acid structure [99]. The CCK-2R (also called the CCKBR) has a higher affinity for gastrin than for CCK, unlike the CCK-1R, and is the most well-characterised gastrin receptor [100, 101]. This 7-transmembrane GPCR (Figure 1.8) sits on the surface of ECL cells, coupled to both a PTX (pertussis toxin)-sensitive (or  $\alpha$ -subunit) and a

PTX-insensitive (or  $\beta\gamma$  subunit) G protein [100]. The latter permits the production of second messengers, inositol 1,4,5-triphosphate ( $IP_3$ ) and 1,2-diacylglycerol (DAG), from phosphatidylinositol biphosphate ( $PIP_2$ ), mediated by phospholipase C $\beta$  (PLC $\beta$ ) [69]. This leads to an increase in intracellular calcium concentration ( $[Ca^{2+}]_i$ ) as does the activation of protein kinase C (PKC) [100, 102]. The PTX-sensitive G-protein leads to activation of all three of the mitogen-activated protein kinase (MAPK) pathways (Figure 1.9) [66, 71, 100].



**Figure 1.8: The structure of the cholecystikinin receptor or CCK-2R**

*The main amino acids of the binding site are the white letters in filled black circles, and the 3<sup>d</sup> cytoplasmic loop is indicated by the arrow (modified from [103]).*

In addition to being expressed on ECL cells, the CCK-2R is also found in many other normal and tumour cells [104]. These include brain cells, monocytes, T lymphocytes and parietal cells, and SCLC, gastric and colorectal cancers [104]. It has been shown to be up-regulated early in carcinogenesis and this often occurs beside up-regulation of gastrin itself [62], in fact in gastric carcinoma expression levels are correlated [99]. Despite this, its role in colorectal and other cancers has not been clarified due to the range of differing results in the literature [105].

Chao *et al.* have shown that the presence of CCK-2R can change immortalised normal colon cells into tumourigenic cells [106]. They stably transfected NCM356 cells with CCK-2R, and found that these

cells grew significantly faster than the vector controls and that they could initiate tumours in nude mice, a trait not shared by the wild-type cells [106].

### 1.7. The signalling pathways downstream of the CCK-2R

The CCK-2R is coupled to many proteins involved in cell signalling [98], via several different pathways. Activation of the CCK-2R can induce Src (sarcoma-inducing tyrosine kinase) to phosphorylate Shc (Src homology domain containing protein) [66]. This allows the small G-proteins, ras and then raf, to be stimulated. These events lead to activation of the three MAPK pathways; the extracellular signal-related kinases (ERKs), the p38 MAPKs and the *c-Jun* NH<sub>2</sub> terminal kinases (JNKs) [102] (Figure 1.9).

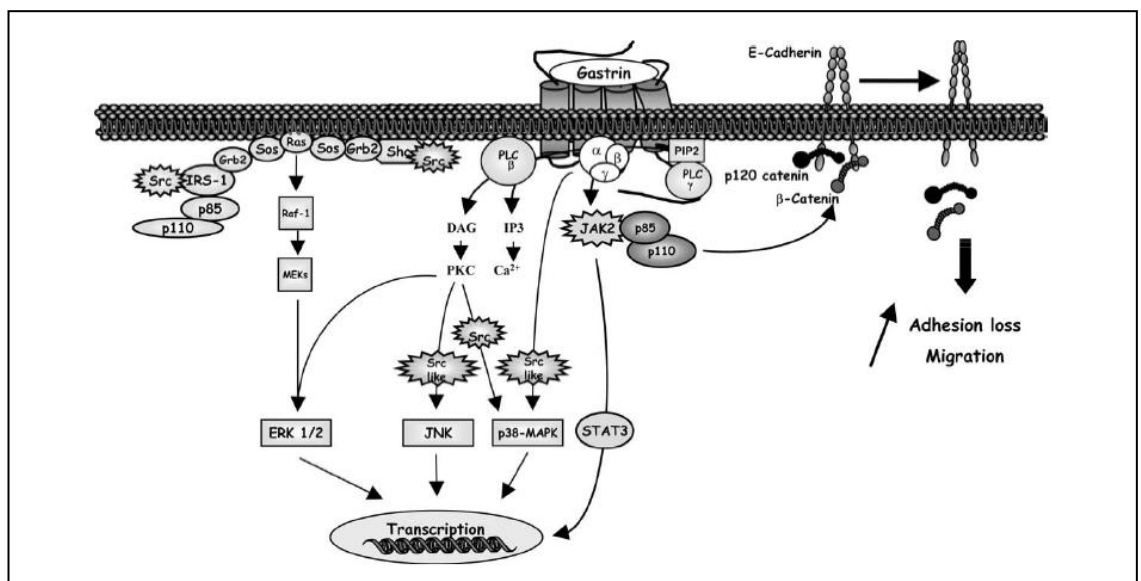


Figure 1.9: The signalling pathways of the gastrin-activated CCK-2R [66]

Each of these pathways lead to the up-regulation of TFs and therefore to the transcription of genes involved in carcinogenic processes. The phosphatidylinositol 3-kinase (PI3K) class I pathway shown above (PIP<sub>2</sub> cleaved to IP<sub>3</sub> and DAG), regulates proliferation, cell adhesion and apoptosis. Specifically the anti-apoptotic protein, Akt, can be activated

via the PI3K pathway, which not only represses apoptosis but also inactivates pro-apoptotic factors [13].

Gastrin can directly stimulate ECL cells to proliferate, however its effects on other cells, such as parietal cells are induced via growth factors in a paracrine fashion [65]. An example of these growth factors is heparin-binding epidermal growth factor (HB-EGF).

#### 1.7.1. HB-EGF

One of the important effects of the constitutively active form of the CCK-2Ri4sv (see Section 1.9.1 below), or of gastrin binding to the wild-type CCK-2R, is the increased expression of HB-EGF (Figure 1.10). This growth factor is produced as a propeptide and cleaved by matrix metalloproteinases (MMP) [107], which are also up-regulated by activation of the CCK-2R [74]. Many different carcinoma cell-lines have been found to contain excessive levels of HB-EGF, and it seems that infection with *H. pylori* can further increase these. This means that the combination of gastrin or a constitutive CCK-2Ri4sv with *H. pylori* infection can considerably increase tumour growth [74]. A further feedback mechanism occurs because *H. pylori* can also cause up-regulation of both the gastrin and CCK-2R genes. This happens in the gastric mucosa in humans and in cultured gastric carcinoma cell-lines.

In the HUVEC (endothelial) cell-line gastrin has been shown to cause tubule formation and therefore promotion of angiogenesis via HB-EGF [108]. Vasoconstriction in tumours can also be caused by the receptor, somatostatin receptor two (SSTR2), [109] allowing hypoxic conditions to persist within the cancer. When this happens gastrin is able to up-regulate hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), causing vascular endothelial growth factor (VEGF) to induce further blood vessel formation. Despite GRP itself having the opposite effect, a GRP

homologue, bombesin, also causes angiogenesis by activating VEGF [81].

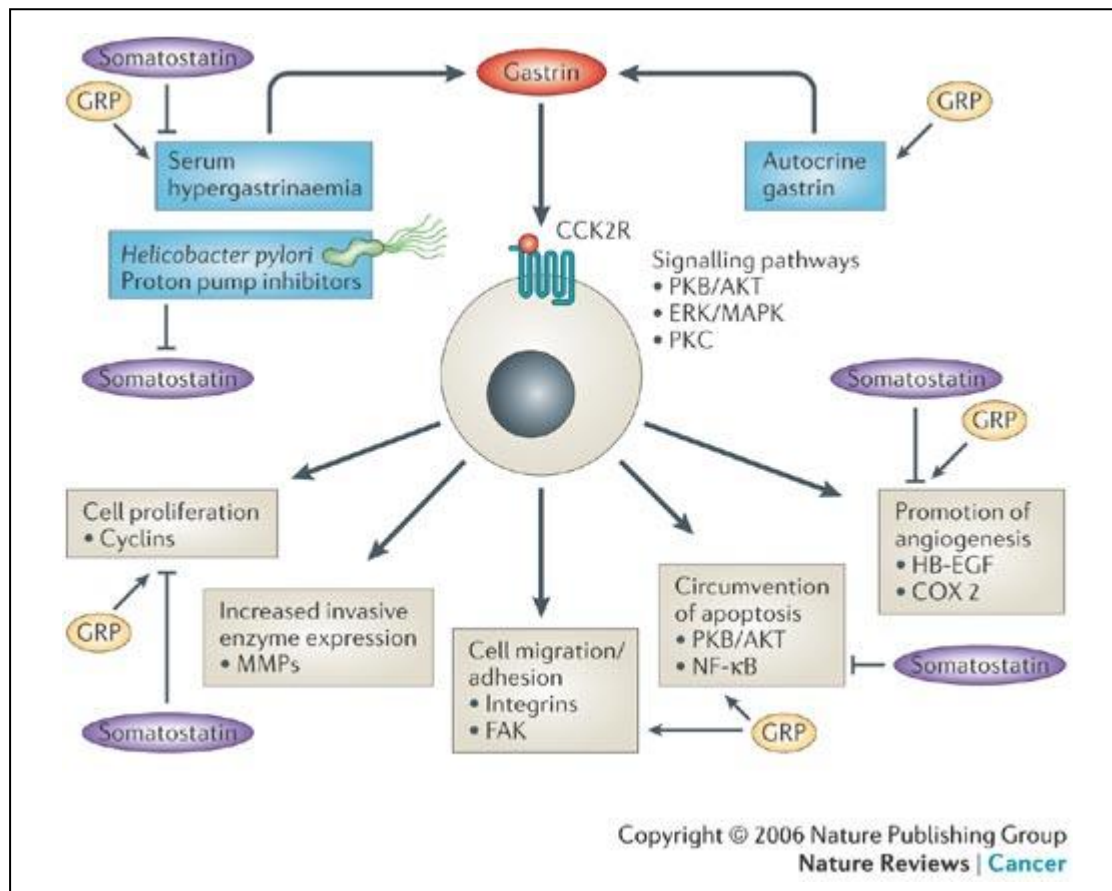


Figure 1.10: The carcinogenic pathways stimulated by gastrin [81]

### 1.7.2. Akt /Protein kinase B

Gastrin has an anti-apoptotic role in cancer (Figure 1.10). Akt, or Protein kinase B (PKB) as it is also known, is an anti-apoptotic protein. It is known that it can down-regulate pro-apoptotic factors as well as playing a direct role in cell survival [13]. These pro-apoptotic factors include Bad, caspase 9 and the transcription factors involved in activation of the cell death ligand, Fas. Gastrin activates Akt by degrading IκBα, which leads to the phosphorylation and inhibition of Bad. Harris *et al.* [13] found that Akt was highly activated in the presence of CCK-2R, when gastrin was applied to three different oesophageal cell-lines, compared with AR42J (rat pancreatic). A high proportion of phosphorylated (active) Akt was found in OE33 cells



transfected with the constitutive CCK-2Ri4sv too, despite no gastrin being present [13]. This indicates that the CCK-2Ri4sv could also be a potent stimulator of oesophageal carcinoma if it is present in these tissues. In addition gastrin can increase the transcription of X-linked inhibitor of apoptosis (XIAP), and in conditions of low somatostatin also leads to high levels of Bcl2 (an anti-apoptotic protein) [81].

### 1.7.3. Catenins

Furthermore, gastrin is able to alter adhesion molecules such as p120, E-cadherin and  $\alpha$ - $\beta$ -catenin allowing a loss of adhesion and therefore greater cell movement [66, 81]. Activation of the CCK-2R activates JAK (Janus kinase) which in turn breaks the interaction between  $\beta$ -catenin and E-cadherin.  $\alpha$ -catenin is also severed from  $\beta$ -catenin cutting the cell's connection to other cells via its actin filaments [66]. During the carcinogenic process the cellular epithelium starts to lose its shape and function, and the combination of all these factors together leads to loss of tissue structure and eventually to cellular motility. At this stage the patient's prognosis is poor, since metastasis is a late stage of any cancer. Two studies support this theory, one showing that gastrin can stimulate invasion of cancerous cells through a membrane [110], and the other an *in vivo* study of the pancreas of ElasCCK2 mice [111].

Bierkamp & colleagues [111] found that pancreatic cells of ElasCCK2 mice stimulated with gastrin proved their hypothesis. This stated that the cells would lose normal morphology, the cell cytoskeleton would be rearranged and that crucial adherens molecules would be modified. They found that  $\alpha$ - and  $\beta$ -catenin and E- and N-cadherin (a pancreatic molecule similar to E-cadherin) were all altered leading to a break-down in cell-cell adhesion. This, along with a change in cell morphology leads to cell motility, allowing metastasis to occur. In a study of gastric epithelial remodelling [110], it was found that gastrin can induce MMP-9

expression in AGS cells and that this leads to cell motility and invasion through a basement membrane.

#### 1.7.4. COX-2

Cyclooxygenase-2 (COX-2) is another factor up-regulated by gastrin [112], and can influence three of the main processes in carcinogenesis; circumvention of apoptosis [113], angiogenesis and invasion of tissues (Figure 1.10). This process has been shown to occur in colon carcinoma cells, via activation of ERK or Akt signalling pathways [37].

### 1.8. Regulation of the CCK-2R

It is assumed that the DNA upstream of the CCK-2R gene start codon contains a promoter/regulator for this receptor. Ashurst *et al.* [114] amplified a 1.7kb section upstream of the rat CCK-2R gene, by classical polymerase chain reaction (PCR), and determined the binding sites for various transcription factors. This was investigated in AR42J (rat pancreatic) and RGM1 (rat gastric) cells and included SP1 and GATA, which, when mutated, reduced basal CCK-2R activity significantly [114]. These two sites were between 196 and 130bp upstream of the CCK-2R start codon, in a similar position to the TF-binding sites found on the gastrin promoter. Their data indicated that rat CCK-2R expression was altered by changes in the microenvironment, for example variation in growth factor concentration [114]. Chao & Hellmich [115] claim that a IFN- $\gamma$  regulatory site is present within the CCK-2R promoter region, causing gastrin to exhibit a pro-inflammatory role. Otherwise knowledge of CCK-2R regulation is limited to protein level up-regulation by gastrin [116, 117], and in cancer cells [62].

The CCK-2R gene may be up-regulated in response to stress conditions such as injury, inflammation and repair of tissues [115].

Levels of CCK-2R gene expression were shown by real-time PCR (qRT-PCR) to be higher in Barrett's metaplasia patients than in healthy control individuals [118]. Similarly the stress condition of serum-starvation caused increased transcription of the promoter constructs made by Ashurst *et al.* [114] in various cancer cell-lines.

## 1.9. Other gastrin receptors

### 1.9.1. CCK-2Ri4sv

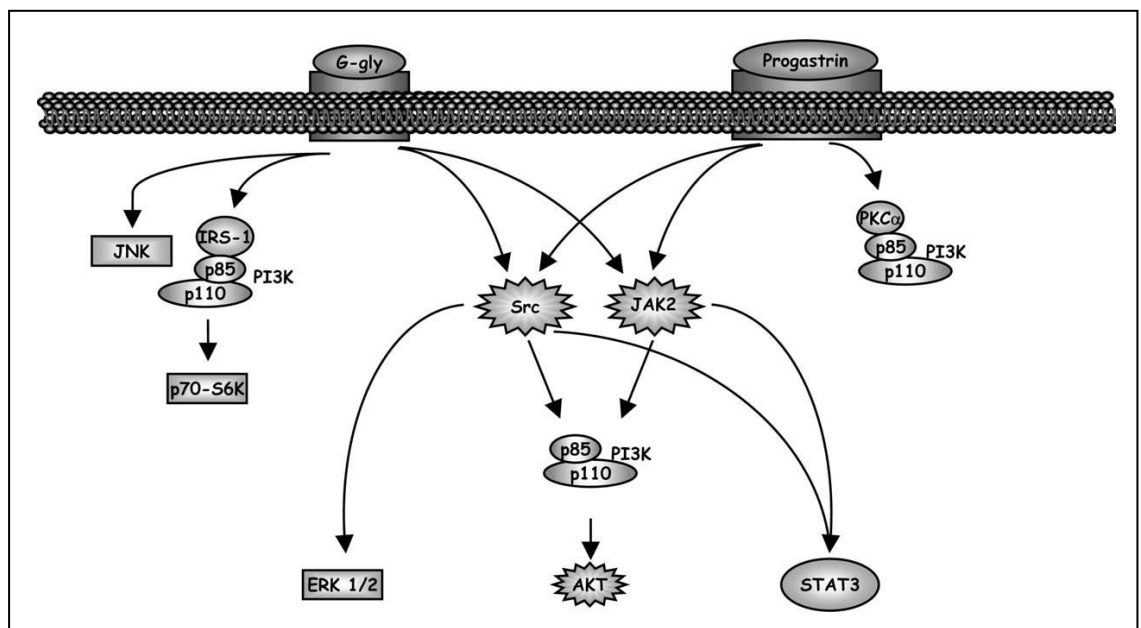
Several variants of the CCK-2R have been sequenced, some with different properties and localisation to the wild type receptor. An example is CCK-2Ri4sv [13, 105] which has retained intron 4 from the original gene. This receptor has been found in colorectal cancer tissues, but crucially not in the surrounding non-cancerous tissue, indicating that it may have a role to play in carcinogenesis. This splice variant happens to show constitutive behaviour, due to its lengthened third cytoplasmic loop domain (Figure 1.8) [105]. This loop is important because it is the point of interaction with G proteins, which in turn cause the signalling effects associated with the CCK-2R. The CCK-2Ri4sv can therefore activate  $\text{Ca}^{2+}$  pathways and cell proliferation cascades without a bound ligand [105].

This modified receptor was also found in colorectal adenomatous polyps, an example of rapidly proliferating but pre-malignant tissue. Again the normal colorectal tissue surrounding the polyps did not contain the CCK-2Ri4sv [101, 105]. This trend is repeated in pancreatic cancer with adjacent tissue bearing only the wild-type receptor [119]. The authors of this work, Ding *et al.*, have investigated this splice variant further; finding that modification of the 3' end of the fourth intron of the CCK-2R gene is critical in its retention. More specifically the splicing factor, U2AF35, was shown to be down-regulated in cancer

tissues, in addition to being polymorphic. However, the latter was found to be insignificant in preventing intron 4 from being removed [119].

### 1.9.2. Other CCK-2R isoforms

Due to the fact that gastrin peptides have only some of the effects of amidated gastrin (Figure 1.11) it is thought unlikely that they work through the normal gastrin receptor, CCK-2R [66, 88]. Progastrin and Gly-gastrin have also been shown to have a biological effect in many cells which do not express the CCK-2R, and to be unimpeded by selective CCK-2R antagonists [83]. It has been hypothesised that there may be another receptor which can be activated by one or both of these intermediaries. It is possible also that gastrin can stimulate certain cancer cells through a receptor other than the CCK-2R [120], or through an isoform of the CCK-2R itself.



**Figure 1.11: The signalling pathways of progastrin and Gly-gastrin, two intermediates in the synthesis of amidated gastrin [66]**

Isoforms of the CCK-2R, other than the CCK-2Ri4sv, have been identified in the literature. These include a truncated form with a deletion in exon 1b [121], a 'long isoform' with a section of the intron 4 spliced into the protein [122] and several frameshift mutation variants

[123]. The first of these, the truncated form, does not appear to have different binding affinities or activity, compared to the wild-type, due to the fact that the mutation occurs in the 5' non-coding region [121]. The long isoform (where the wild-type CCK-2R is the 'short isoform' due to loss of intron 4), is similar to CCK-2Ri4sv in that the third cytoplasmic loop is enlarged, but the authors did not study the role of these receptors [122]. Some of the variants with frameshift mutations have been found to be shortened due to a premature stop-codon, and all were found in tumours with microsatellite instability [123].

#### 1.9.3. Heterodimerisation of CCK-1R and CCK-2R

An alternative explanation for the fact that there are some effects of gastrin peptides which cannot be successfully attributed to the CCK-2R, yet no other CCK receptor subtypes have been discovered, is that the CCK-2R forms either homodimers, or heterodimers with the CCK-1R [124]. This has mostly been studied in the brain, where Cheng & colleagues [124] suggest that most of the cells where these two receptors co-localise are tumour cells, therefore suggesting a role for these heterodimers in gastrin peptide-induced tumour progression.

#### 1.9.4. Annexin II

Singh *et al.* [125] identified a 33-36 kDa protein which seemed to bind gastrin-like peptides with much higher affinity than CCK-8 (Figure 1.12). They discovered that this protein correlated to annexin II, and that this is a receptor which can at least partially mediate the growth factor effects of progastrin and other gastrin intermediates [125, 126]. Annexin II was previously known to bind acid phospholipids and actin and to be present in actively replicating cells [126]. It has now been found on colon carcinoma cells, and indeed many other human cancer cells, which is in keeping with its role in rapidly proliferating cells. It is thought that when progastrin binds to and activates annexin II, NF- $\kappa$ B and p38

MAPK can be activated, causing proliferation of tissues (Figure 1.12) [126].

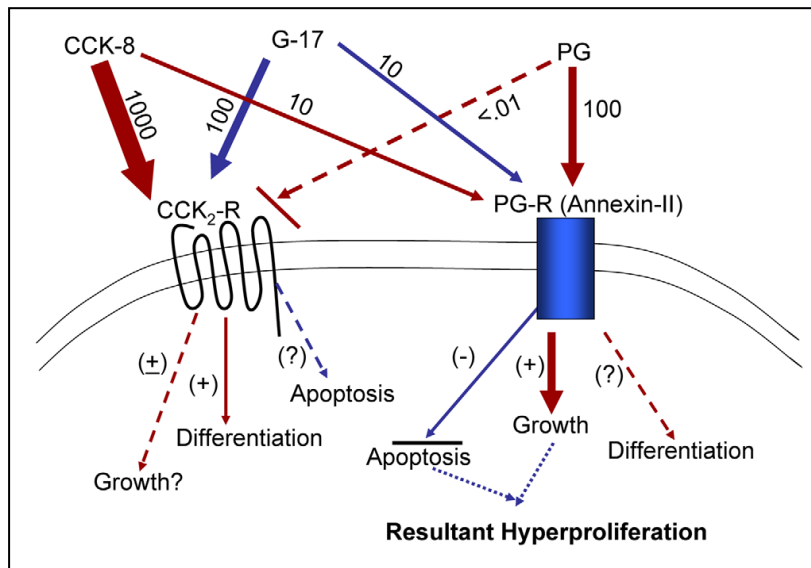
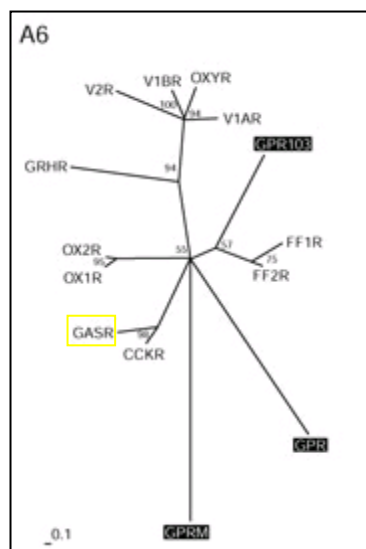


Figure 1.12: Hypothetical model of the binding and down-stream effects of annexin II [126]

### 1.10. Other GPCRs in relation to the CCK-2R

In order to better understand the CCK-2R, other GPCRs can be studied to discover similarities and to investigate better techniques to observe the CCK-2R. GPCRs are very important to science since they make up 30% of all licensed therapeutic drug targets. The CCK-2R is a peptide receptor in the Class A, or Rhodopsin-like, GPCRs [127]. This is a very large family with 19 subgroups, of which CCK-2R is in subgroup A6, along with the hypocretin (orexin) receptors (OXRs), vasopressin receptors (VRs) and the gonadotropin releasing hormone receptor (GnRH-R; Figure 1.13) [127].



**Figure 1.13: Phylogenetic tree showing subgroup A6, of the peptide class A G-protein coupled receptors (GPCRs)**

*Abbreviations:*

CCKR	Cholecystokinin 1 receptor
FF1/2R	Neuropeptide FF 1/2 receptor
GASR	Gastrin/Cholecystokinin 2 receptor
GRHR	Gonadotropin releasing hormone receptor
OX1/2R	Orexin (hypocretin) receptor type 1/2
OXYR	Oxytocin receptor
V1A/1B/2R	Vasopressin 1A/1B/2 receptor

The CCK-2R is indicated by a yellow box and orphan receptors are shaded. Adapted from Figure 4 and Additional data file 1 of Joost & Methner [127].

### 1.10.1. Orexins

According to Figure 1.13, the OXRs are the closest phylogenetically to the CCK receptors. Orexins also have two distinct receptors, OX-1R and OX-2R, where the former binds orexin-A, and the latter orexin-A and -B with equal affinity [128, 129]. This is a similar set-up to the CCK receptors; where CCK and gastrin are the ligands. The OXRs were first found throughout the human CNS [128], but are now known to be present in the GI tract [130], and their ligands have roles in appetite, cardiovascular, neuroendocrine and temperature regulation.

Spinazzi *et al.* [129] investigated the expression of both OXRs in normal and tumour adrenocortical cells, and found that qRT-PCR showed gene expression similar to that of the protein expression shown by western blotting. They also showed an up-regulation of the receptors in the tumour adenoma tissue, compared to the normal [129].

### 1.10.2. Neuropeptide FF receptors

These receptors bind two ligands; NPAF (A-18-F-amide) and NPFF (F-8-F-amide) [127]. The neuropeptide FF 2 receptor (FF-2R) has been shown to have alternative splice variants, namely one which translates

exon 1 called the NPGPR [131], which is similar to the differences between the CCK-2R and the CCK-2Ri4sv isoform. FF-2R has been shown to be expressed in the hypothalamus by qRT-PCR, another similarity to the CCK-2R, although the detection problems in qRT-PCR were not replicated with this GPCR [131]. This receptor is involved with nociception and morphine-tolerance.

#### 1.10.3. Gonadotrophin releasing hormone receptor

Another GPCR in the same subfamily as CCK-2R is the GnRH-R, which is expressed in the brain and ovaries and has also been found in pancreatic tumours [132]. In the ovary, where GnRH-R and its ligands, GnRH-I and -II, have been shown to be expressed by qRT-PCR, its role is to be a progesterone regulator [133]. Also, GnRH-R agonists are used clinically in prostate cancer, to reduce testosterone secretion and therefore reduce tumour size [132, 134].

#### 1.10.4. GPCRs and the CCK-2R

While the GPCRs examined here have very similar roles and expression patterns to the CCK-2R, they do not seem to be comparable in terms of detection. They are all expressed in the brain and other vital organs, some even in tumour cells, and they are all peptide, or hormone, receptors, however, they do not seem to share the same transcriptional regulation as the CCK-2R, which can be found easily at the protein level, but not so at the gene level.

### 1.11. **CCK-2R antagonists**

There have been many CCK-2R antagonists described, although not all of them are specific enough to eliminate CCK-1R blockade [135]. In 1998, several benzodiazepine-derived antagonists were being used, such as L-365,260 and a second generation benzodiazepine, L-740,093



[135]. The latter has a higher affinity for CCK-2R and also has better solubility in water. Many CCK-2R antagonists are used now, including a few which have been found to be useful in a clinical setting. For example, YM022 reversed the increase in CCK-2R effected by *H. pylori* infection [72]. Also Gastrozole (JB95008), a new CCK-2R antagonist has been trialled as a drug for pancreatic cancer [21]. It has activity against gastrin-induced cell proliferation and, although not orally bioavailable, via intravenous administration has been successful in increasing survival without toxicity.

A new orally available CCK-2R antagonist, Z360, has also been developed and shown to compete with amidated gastrin in NIH3T3 and OE33 cells [72]. The drug has been shown to reduce angiogenesis in C170HM2 *in vivo* models, and pancreatic xenograft models, and to inhibit metastasis of MGLVA-1 cells. Z360 is known to be highly selective for the CCK-2R and is now in clinical trials for patients with advanced pancreatic cancer [72, 136].

Short interfering RNAs (siRNAs) are beginning to be used for this purpose too, and the use of these against CCK-2R has been shown to inhibit proliferation and promote apoptosis in gastric cancer cells [137].

#### 1.11.1. CCK-2R antagonists as a method of detection

These inhibitors of the CCK-2R are extremely important in determining the expression of the receptor in cancer cell-lines, because CCK-2R mRNA is very difficult to detect by PCR [72]. Specific antagonists have been used to this effect in oesophageal [13], colorectal [138] and gastric [74] carcinoma cells, but an explanation for the low level gene expression is still required.

YM022 was used to block the CCK-2R in oesophageal cells (OE19 and OE33), which caused basal phosphorylation of Akt to drop, as opposed to the addition of gastrin, which caused an increase in phosphorylation [13]. In AGS cells YM022 was utilized to determine the role of the CCK-2R in HB-EGF expression. Both basal and *H. pylori*-induced HB-EGF expression could be reduced when the antagonist was added [74].

Colucci *et al.* have shown that blockade, by L-365,260 and GV150013, of the CCK-2R can reverse the growth-promoting effect of gastrin in HT29 (colorectal) cells, and also reduce COX-2 amplification induced by the presence of gastrin [138]. HT29 cells were also found to show this effect, when JMV 320 was used to inhibit the gastrin receptor [139]. Ishizuka *et al.* also found that JMV 320 blocked gastrin-stimulated proliferation or inhibition of LoVo or HCT116 cells, respectively. However, the effect is not universal in colonic carcinoma cell-lines, since the inhibitor had no effect on gastrin-stimulated growth of COLO 320 cells [139].

### 1.12. siRNA

An alternative method for blocking the CCK-2R is to use siRNA, specific for the receptor, to knock-out expression altogether [140, 141]. This method has been used to down-regulate gastrin [82], in order to investigate the role of endogenous gastrin alone. This was very successful and proved the technique to be extremely specific, and effective at reducing gastrin expression in several GI cell-lines [82].

siRNA can be designed from the sequence of the gene in question using algorithms e.g. those available on the websites: Dharmacon, Genscript and siSEARCH. These algorithms apply a set of rules, which differs for each algorithm (an example is found in [140]), to find specific 21 nucleotide sequences and then the results are analysed. Factors

such as position in the gene, GC content and seed frequency (number of modifications necessary) are taken into account.

Since this method is very specific, it is likely that using it to knock-out the CCK-2R will be more successful than the antagonists described above. With this method it is assumed that only the CCK-2R will be down-regulated since a part of its mRNA sequence is being targeted, as opposed to a drug which may affect other receptors or proteins in the target cell. In the gastrin siRNA experiment the method was even able to overcome up-regulation by EGF, and so it may be possible to reverse the up-regulation of CCK-2R seen in cancer cells [82]. In short CCK-2R siRNA may provide a better tool for understanding the role of this receptor in cancer progression.

### **1.13. Hypothesis and aims**

The hypothesis for this project is that the CCK-2R plays a role in the progression of cancer and that its effects may be up-regulated during stress conditions such as hypoxia and nutrient deficiency. It may play a vital role in the expansion or maintenance of a specific subset of cells within the tumour; possibly the cancer stem cells.

The current qRT-PCR method used to detect gene expression of the CCK-2R is not sensitive enough to reveal endogenous expression in cell-lines where CCK-2R protein has been recognized previously [13]. A new method, called Linear-After-The-Exponential-PCR; LATE-PCR [142], has been hypothesised to increase sensitivity above that of normal PCR by altering primer concentrations to give linear product. This will be tested against the CCK-2R Taqman qRT-PCR currently in use in the laboratory, to establish whether lower concentrations of mRNA can be detected. Perhaps this assay will demonstrate CCK-2R gene expression in cancers where it has not been previously identified.

In order to validate the LATE-PCR assay siRNAs to the CCK-2R will be designed and tested on a range of cancer cell-lines *in vitro*. These cells will also be stimulated with a range of factors, including hypoxia, serum-starvation and cytotoxics to investigate the effect of a stress environment on the CCK-2R. A 3D culture model will be used to select for stemness in the cell population and therefore determine if the receptor has any effect on the expansion or maintenance of the cancer stem cell subset. If up-regulation of the CCK-2R is observed in these situations the mechanism of action will be challenged using knock-down or inhibition of the receptor.

Ultimately if the signalling mediated via the CCK-2R can be removed from tumour cells *in vivo*, this should reduce the carcinogenic effects of gastrin. Therefore the CCK-2R is a potential therapeutic target.

## Chapter 2    Materials and Methods

## 2.1. Cell culture

### 2.1.1. Cell growth

The cells used in this study (Table 2.1) were maintained at 37°C in a humidified CO<sub>2</sub> incubator and cultured in normal growth medium (Table 2.5), in 75cm<sup>2</sup> flasks. All cell-lines were adherent cells, with the exception of H209. Transfected cell-lines had their growth medium supplemented with 2µl geneticin per ml medium to select for transfected cells.

### 2.1.2. Sub-culture of cells

The cells were split when they reached 60-100% confluency (judged under a microscope at low power). The medium was aspirated away and 3ml of either 0.025% EDTA or 0.25% Trypsin-EDTA, depending on cell-line, was used to detach the cells from the flask. The cells could be harvested after 5-10 min and were pelleted in the centrifuge at 1500rpm for 5 min. The medium plus EDTA was aspirated away and the cells re-suspended in a suitable volume of normal culture medium. Lavaging through a green 10ml gauge needle was used to separate clumps of cells, then the cells were counted and a fraction returned to the flask in 15ml of normal growth medium.

### 2.1.3. Cell counting

Trypan blue was made up as a 4:1 mixture of 0.2% trypan blue to 4.25% NaCl. Equal volumes of cells and trypan blue were mixed and ~10µl pipetted onto a Neubauer haemocytometer covered with a coverslip. With the coverslip on the chamber of the haemocytometer a primary square holds 1mm<sup>3</sup> of liquid. The cells in this volume were counted and the number (an average of several) was multiplied by 2x10<sup>4</sup> to give a concentration in cells/ml.

2.1.4. Table of cell-lines used in this project

Cell line	Origin of cell line (all adherent unless stated)	Transfected variants used	Medium grown in
A549	Human male non small cell lung cancer (NSCLC) [143] Mutated genes include CDKN2A and KRAS [144] Isolated from explant culture of lung carcinoma tissue [145]		RPMI
AGS	Human female gastric adenocarcinoma [143] Mutated genes include PIK3CA and KRAS [146] Isolated from fragments of a tumour removed from a patient who had received no therapy [145]	CCK-2R, VC	RPMI
AR42J	Rat exocrine pancreatic tumour [143]		RPMI
BT4	Paediatric giant cell high grade (IV) glioblastoma later revised to malignant glioneuronal tumour (named GBM1 in [45])		DMEM
C170HM2	Hepatic invasive human colorectal, selected from the parent cell-line; C170 [147]		RPMI
CA20948	Rat pancreatic [148]		RPMI
EKVX	Human lung adenocarcinoma (NSCLC) [149]		RPMI
(NCI-) H209	Human male small cell lung cancer (SCLC) derived from bone marrow metastatic site (grows in suspension) [143] Mutated genes include RB1, and isolation was from bone marrow [145]		RPMI
(NCI-)H23	Human male NSCLC [145]		RPMI
HCT116	Human male colonic adenocarcinoma [150] Mutated genes include CDKN2A and BRCA2 [144]		RPMI
HGT-1	Human male gastric adenocarcinoma from the body of the stomach [151]		RPMI
HOP-62	Non small cell lung cancer [152] Mutated genes include CDKN2A and KRAS [144]		RPMI
HOP-92	Non small cell lung cancer [152]		RPMI
HT29	Human female colonic adenocarcinoma [143] Mutated genes include APC and BRAF [144] This cell-line has been seen to exhibit microvilli and expresses the oncogenes c-myc, and K-ras [145]		RPMI
LoVo	Human male colonic adenocarcinoma, at Dukes type C [143]		LoVo medium
MCF-7	Human female breast carcinoma [143]		RPMI
MDA-MB-453	Human female breast, mammary gland metastatic carcinoma [145]		RPMI
MGLVA-1	An ascetic variant of the gastric cell-line MKN45G [153]		RPMI
NIH3T3	Mouse embryonic fibroblast [150]	CCK-2R	RPMI
OE19	Human male adenocarcinoma of the gastric cardia/oesophageal gastric junction, pathological stage III [150]		RPMI
OE33	Human female Barrett's metaplasia derived oesophageal adenocarcinoma, pathological stage II [150]	CCK-2R	RPMI
OLIG1	Paediatric glioma [45]		DMEM

OVCAR-8	Human ovarian [149]		RPMI
PAN-1	Pancreatic (in house)		RPMI
PANC-1	Human male pancreatic epithelial, ductal, carcinoma [143]		RPMI
SKBR3	Human female breast, mammary gland (pleural effusion), adenocarcinoma [150]		RPMI
SK-OV-3	Human female ovarian adenocarcinoma [143]		RPMI
ST16	Human gastric (in house)		RPMI
SW620	Human male colon adenocarcinoma [149] Mutated genes include KRAS and APC [144] Isolated from a recurrence of the malignancy, which resulted in a wide-spread metastasis, taken from a lymph node [145]		RPMI
T47D	Human female breast, mammary gland (pleural effusion), ductal carcinoma [150]		RPMI
UACC-257	Human melanoma [152]		RPMI
U251	Human glioblastoma/astrocytoma [154] Mutated genes include CDKN2A and PTEN [144]		RPMI
U373-MG	Human male neuronal glioblastoma (brain; astrocytoma) at grade III [143] Mutated genes include PTEN and AKT1 [146]		RPMI
U87-MG	Human female neuronal glioblastoma (brain; astrocytoma) at grade III [143]		RPMI
ZRS51	Human female breast, mammary gland metastatic site: ascites, ductal carcinoma [150]		RPMI

**Table 2.1: Cell-lines used in this project**

#### 2.1.5. Freezing cells

The cells were harvested and counted. Once counted the cells were pelleted and re-suspended in freezing medium to a concentration of  $2 \times 10^6$  cells/ml. 0.5ml was aliquoted into each cryogenic tube and labelled appropriately. The tubes were moved immediately to the  $-70^{\circ}\text{C}$  freezer and to liquid nitrogen storage after at least 24 hours.

#### 2.1.6. Thawing cells

15ml of medium was added to a  $75\text{cm}^2$  flask, labelled and put at  $37^{\circ}\text{C}$  for 30 min. The frozen aliquot of cells was taken straight from liquid nitrogen storage to a  $37^{\circ}\text{C}$  water bath and swirled gently. Once 90% defrosted the tube was sprayed with 70% ethanol and the contents removed into the pre-warmed flask and incubated overnight. The cells



were re-fed with normal growth medium after 24 hours to remove traces of DMSO.

#### 2.1.7. Transfection with plasmid DNA in normoxic/hypoxic conditions

CCK-2R promoter plasmids were used to investigate the regulation of this receptor. The cells were plated at  $2 \times 10^5$  or  $8 \times 10^4$  cells/ml in the middle 8 wells of a 24-well plate 24 hours before the transfection was to be carried out. 200  $\mu$ l of medium was added to the outside wells of a 24-well plate, in order to avoid infection. Mastermixes of 5  $\mu$ l of 100ng/ $\mu$ l plasmid of interest DNA and 1  $\mu$ l of 100ng/ $\mu$ l  $\beta$ -galactosidase plasmid DNA with 44  $\mu$ l of OptiMEM 1 medium were made up. In a separate tube 2  $\mu$ l of lipofectamine was added to 48  $\mu$ l of OptiMEM 1 medium for every well to be transfected plus one, and this was left to incubate for 5 min. The lipofectamine mix (50  $\mu$ l per well) was then added to the diluted DNA and incubated for 20 min at room temperature.

The plated cells were assessed for confluency, which should have been 50-80%, and then the medium was aspirated from the wells and replaced with 500  $\mu$ l fresh normal growth medium. The transfection complex was dropped into the well slowly while on a rocking platform to ensure even distribution. The plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 6 hours after which the transfection medium was aspirated away to be replaced with 1ml serum-free medium. The plates were placed back in the incubator overnight.

24 hours after the medium change, half the plates were placed in the hypoxia chamber, while half of them remained in the incubator (in normoxia). After 16 hours the cells were harvested, either for luminometer assay or RNA extraction.

#### 2.1.8. Harvesting transfected cells for luminometer assay

Firefly luciferase expression was used as a measure of promoter activity, with  $\beta$ -galactosidase expression as the control, and these values were assessed using a luminometer. Each plate was aspirated to remove all medium from the cells, and then 100 $\mu$ l of PBS was added to each well to wash the cells. This was aspirated away before 100 $\mu$ l of 1x PLB per well, was added. The plates were rocked gently for 20 min to loosen the lysates from the wells then these were transferred to labelled eppendorf tubes and frozen at -70°C, ready for luciferase and  $\beta$ -galactosidase assay on the luminometer.

#### 2.1.9. Harvesting transfected cells for RNA extraction

If gene expression was to be assessed, RNA extraction was the first step. The medium was aspirated away from each well and cells were treated with 500 $\mu$ l TRI reagent. They were incubated for 5 min and then transferred to eppendorf tubes for RNA extraction with half volumes.

#### 2.1.10. Sphere forming

Sphere-forming was used to quantify the stem cell content of a cell-line population. The cells were harvested as above, then re-suspended;  $\sim 1 \times 10^6$  cells in 12ml of stem cell medium, and plated into one 6-well plate at 2ml/well. A monolayer cell plate was also plated as a control in normal growth medium (1000 cells/plate due to growth restrictions over ten days). Cells were re-fed with EGF and FGF every three days (at day four, seven and ten). If YM022 was added to the plate, this was also re-fed at a concentration of  $10^{-8}$  every three days.

#### 2.1.11. Harvesting sphere cultures

The spheres often floated in the medium, meaning they needed to be harvested and centrifuged to remove the medium, before normal RNA

extraction or luminometer assays or live cell staining could be carried out.

However, for paraffin wax embedding the spheres needed to stay together so they were carefully transferred into a universal and left to settle for 2 hours at 4°C. Once settled the medium was carefully removed, using the aspirator and then a pipette, and 50µl of 4% PFA was added. The spheres were incubated at room temperature for 10 min, before as much supernatant was removed as possible. A small eppendorf had been prepared earlier with 200µl of set 1.5% agarose in it. 200µl of cooled liquid 1.5% agarose was pipetted into the eppendorf, and the spheres were carefully pipetted into this (using a cut tip) and mixed once. The agarose was left to set.

The end of the eppendorf was cut with a scalpel to expose the agarose plug with the spheres in it. This plug was carefully pushed into a bijou full of formal calcium, and left to fix overnight. The plug was placed into a processing cassette, and put through the processor, before embedding in wax. Sections were cut from this sample with the microtome, placed on polysine slides and stained.

#### 2.1.12. MTT assay

The IC<sub>50</sub> of a drug in a particular population of cells can be assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at 1x10<sup>4</sup> cells/well in 100µl of normal growth medium in the middle 60 wells of a 96-well plate. The outer wells had 100µl of normal growth medium added to them to prevent dehydration. The plate was incubated overnight at 37°C in a 5% CO<sub>2</sub> incubator, before treatment with a 1/2 dilution series of cisplatin (from 200-0.3µg/ml). These were added in 100µl aliquots to five wells each and, with 2 sets of medium-only wells, made up 12 conditions per plate.

This was added to the medium already there in order to disturb the cells as little as possible. The cells grew in the incubator for 48 hours before MTT solution was added, 50µl/well. After a 4 hour incubation at 37°C the plates were aspirated carefully into a waste bottle (for incineration, not sink disposal) and 75µl of DMSO added to each well, plus the five control wells with no cells. The plate was read at 550nm and the results analysed in GraphPad Prism in order to ascertain the IC<sub>50</sub>.

## **2.2. siRNA**

### **2.2.1. siRNA design**

siRNAs were designed to target different sites within the receptor mRNA, using a number of different algorithms available on the internet (Dharmacon, Ambion, siSEARCH and Genscript). Each yielded a list of potential siRNAs, which were ranked due to several factors. For example, on Dharmacon's website there is a column for 'Low seed frequency' which, if 'Yes', means that the siRNA is less likely to bind to other genes in the genome of that organism and act as a micro RNA (miRNA).

### **2.2.2. siRNA synthesis**

Each siRNA was ordered as two oligonucleotides, a sense and an antisense strand. These had to be annealed into a double-stranded molecule using the Ambion Silencer siRNA construction kit. To prepare the transcription template, 2µl of a 100µM solution of each strand were placed in separate tubes, along with 2µl of T7 promoter primer and 6µl of DNA hyb buffer in each. Both tubes were heated to 70°C for 5 min, and then left at room temperature for 5 min more. Klenow DNA polymerase mix was added to each tube consisting of 2µl of 10X Klenow reaction buffer, 2µl 10X dNTP mix, 4µl nuclease-free water and 2µl Exo-Klenow. The tubes were mixed by gentle pipetting, then centrifuged and incubated at 37°C for 30 min.

The next step was the double-stranded RNA synthesis; 2µl of each of the previous mixtures were added to two new tubes along with 4µl nuclease-free water, 2µl 10X T7 reaction buffer, 10µl 2X dNTP mix and 2µl of T7 enzyme in each. These transcription reactions were mixed gently, centrifuged and incubated at 37°C for 2 hours. The sense and antisense reactions were then combined into one tube and incubated at 37°C overnight.

The final stage prepared and purified the siRNA. The tube containing the annealed strands from above had 6µl digestion buffer, 48.5µl nuclease-free water, 3µl RNase and 2.5µl DNase added to it. This was mixed gently then incubated at 37°C for 2 hours, before the concentration was measured to ensure it was appropriate for transfection (~20µM).

### 2.2.3. Transfection with siRNA

The cells were plated at  $5 \times 10^4$  cells/ml in a 6-well plate, 24 hours before the transfection was to be carried out. Mastermixes of 4µl of 25µM siRNA with 10µl of siPORT Amine were made up to a total volume of 200µl per well with OptiMEM 1 medium. The medium and siPORT Amine were mixed, vortexed and incubated for 30 min at room temperature. Then the siRNA was added and left to incubate for 20 min, also at room temperature. The medium was aspirated from the wells and replaced with 800µl fresh normal growth medium. The transfection complex was dropped into the well slowly while on a rocking platform to ensure even distribution. The plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 6 hours then 1ml medium was added to each transfected well. The plates were then put back in the incubator overnight.

24 hours after transfection the cells were harvested either for RNA extraction or, if fluorescent siRNA was used, for cytopins.

#### 2.2.4. Reverse transfection with siRNA

This method was used for non-adherent cells such as H209. 1.5µl of siPORT Amine was added to 23.5µl of OptiMEM 1 per well in a mastermix and incubated at room temperature for 10 min. 0.75µl of the correct siRNA was added to 24.25µl of OptiMEM 1, per well, and then 25µl of the Amine mix was added to each tube. This was incubated for 10 min and then 50µl was spread around the bottom of an empty well. Each 24-well plate had its outer wells filled with 200µl of normal growth medium.  $8 \times 10^5$  cells in 450µl of normal growth medium were overlaid into each middle well and the plate placed on a rocking platform for 5 min. The plate was incubated at 37°C for 6 hours and then 1ml of normal growth medium was added to each transfected well. The plate was then left in the incubator at 37°C overnight.

#### 2.2.5. Harvesting siRNA-transfected cells for RNA extraction

If the cells grew in suspension they were transferred to eppendorfs, spun down at maximum speed for 3 min, aspirated, then placed in 500µl TRI reagent. Adherent cells were treated with 1ml TRI reagent per well after the medium was aspirated away. The cells were incubated for 5 min and then the lysates were transferred to eppendorf tubes for RNA extraction.

#### 2.2.6. Harvesting siRNA-transfected cells for cytopins

In order to see the location of the fluorescent TAMRA-siRNA within the cells, they were put on slides, nuclear-stained with DAPI, and observed through a microscope. Suspension cells were placed in eppendorfs, pelleted, aspirated, washed in 100µl PBS and centrifuged again.

Adherent cells had their medium aspirated away and the cells were also washed in 100µl PBS. Either way the PBS was aspirated, 200µl of 0.4% formalin was added to each well and the plate left on a rocking platform for 20 min. If still in the plate, the lysate from each well was transferred to an eppendorf, making sure all the cells had been removed from the bottom of the well.

## **2.3. Histology**

### **2.3.1. Cytospins**

A volume containing  $1 \times 10^4$  cells was added to an assembled cytospin module with labelled slide. These were spun in the cytospin for 5 min at 1500rpm. The modules were disassembled and the slides left in a dark place to dry for 30 min. The slides were put into a holder and briefly washed in PBS. 100µl of the counterstain, Hoechst, at  $0.5 \mu\text{g}/\mu\text{l}$ , was placed over the circle of cells on the slide for 5 min. The slides were again washed briefly in PBS in their holder and then dried slightly. One drop of either Citifluor or ProLong was placed on a coverslip (one per slide) and the slide was pressed into this, with the cells underneath the coverslip. The slides were then visualised straight-away if in Citifluor, or left overnight first if in ProLong, under a Leica fluorescent microscope, with DAPI (filter A) and Texas Red (filter TX2).

### **2.3.2. Live cell staining**

This method was used to investigate protein expression in cells. All centrifugation steps were carried out at 4000rpm for 2 minutes at  $4^\circ\text{C}$ , and HEPES buffer was used instead of PBA to improve cell viability. The cells were harvested from a  $75\text{cm}^2$  flask or 6-well plate (sphere and monolayer samples), lavaged if necessary, and counted.  $5 \times 10^5$  cells were put into an eppendorf, centrifuged and the supernatant removed by tipping up the tube. The pellet of cells was re-suspended in the small amount of medium still present, and washed in 500µl of HEPES buffer.

The cells were centrifuged again and the supernatant poured away, before re-suspension and a further wash with 500µl HEPES buffer.

When the cells had been re-suspended in the residual HEPES buffer, 300µl of a 1:10 dilution of goat serum (or mouse serum for CD44 staining) was added to the tube as a blocking agent. The cells were kept at 4°C in the dark to incubate for one hour. The cells were then spun, the block removed and the pellet re-suspended before washing with 500µl HEPES buffer twice. When the cells had been re-suspended after the last wash, 5µl of a 1:5 dilution of the primary antibody was added. This primary antibody was rabbit anti-CCK-2R, mouse anti-CD133 or mouse universal antibody (or mouse anti-CD44 conjugated or the isotype control). Nothing was added to the CCK-2R negative control at this point; a species-matched universal control was used in initial experiments to show specificity of the primary antibody, however it became unavailable for later experiments. The cells were then incubated for one hour at 4°C in the dark.

500µl of HEPES buffer was added to the tubes before they were spun in order to remove the excess primary antibody. The cells were then washed two times in 500µl of HEPES buffer, before adding 300µl of a 1:1000 dilution of the goat anti-rabbit FITC-labelled secondary antibody to the re-suspended pellet (the CD44 labelled cells were fixed at this point). Again the cells were incubated at 4°C in the dark for one hour. After this the cells were spun to remove the excess secondary antibody, washed two times in HEPES buffer and re-suspended in 300µl of the fixative, 0.5% formaldehyde. The fixed samples could then be stored at 4°C in the dark, ready for flow cytometric analysis.



### 2.3.3. Flow cytometry

The two secondary antibodies used and the conjugated primary CD44 antibody were labelled with FITC. Flow cytometric analysis was run in clear tubes on the Coulter Altra Flow Cytometer in the flow cytometry facility, QMC. A programme was developed to detect FITC expression in 10,000 cells from each sample.

### 2.3.4. Paraffin staining

Paraffin staining (or immunohistochemistry, IHC) was carried out for CCK-2R (and  $\beta$ -catenin) in order to locate these proteins within the spheres. The sections were dewaxed in 2x xylene baths, for 5 min each, and then rinsed in 2x 100% ethanol for 1 min each. The block consisted of 0.6% (1%) hydrogen peroxide in methanol for 10 min (15 min), followed by 2x 100% ethanol baths, and a tap water (distilled water) bath for 1 min each. The slides for CCK-2R were microwaved in citric acid for 15 min at 98°C, and quenched in running tap water followed by PBS for 1 min, whereas those for  $\beta$ -catenin were not. The sections were blocked in 20% rabbit (swine) serum at room temperature for 30 min (15 min), then rinsed for 3 min in 2x PBS. The primary antibody, goat anti-human CCK-2R (rabbit anti-human  $\beta$ -catenin) was then added at a 1 in 50 (1 in 100) dilution in PBS to the positives. The negative controls had goat (rabbit) serum at 1/3000 (1/2000) added to them. These were incubated overnight at 4°C (at RT for 60 min) in a humid box.

The sections were then rinsed 2x in PBS for 3 min, and incubated with the secondary biotinylated antibody, rabbit anti-goat (swine anti-rabbit), at a 1/300 dilution in PBS. The sections were rinsed again in 2x PBS for 3 min, before incubation for 30 min (20 min) with ABC at room temperature. 2x rinses in PBS for 3 min were followed by incubation with AEC for 30 min (DAB for 5 min) at room temperature. The rinse

was 2x distilled water (1x running tap water) for 1 min, after which the sections were counterstained with haemalum for 5 min (1 min). The sections were rinsed in running tap water for 1 min.

The CCK-2R slides were placed in distilled water for 1 min before coverslipping with VectaMount and sealing with nail varnish. The  $\beta$ -catenin slides were dehydrated in 3x 100% ethanol baths for 1 min each and cleared for 3 min in 2x xylene baths. DPX was used to mount these coverslips before air-drying overnight.

## **2.4. Molecular biology**

### **2.4.1. RNA extraction**

The first step in demonstrating gene expression in cells involved the extraction of RNA. An eppendorf containing a pellet of  $5 \times 10^5$  cells was taken from the  $-80^\circ\text{C}$  freezer and 1ml of TRI-reagent added to it. This was used to re-suspend the pellet and then left to incubate for 5 min. A control tube was made without a cell pellet. 200 $\mu\text{l}$  of chloroform per ml of TRI reagent was added to the tube, which was shaken vigorously. This was incubated at room temperature for 15 min before centrifuging at 13,000 rpm for 15 min at  $4^\circ\text{C}$ . The aqueous phase, containing the RNA, was removed into a fresh tube containing 500 $\mu\text{l}$  of isopropanol and the tube inverted to mix. This was left at room temperature for 10 min before centrifuging at 13,000 rpm for 10 min at  $4^\circ\text{C}$ . The supernatant was aspirated and the pellet washed in 1ml of 70% ethanol. The tube was centrifuged at 13,000 rpm for 5 min and the supernatant again removed. The pellet was left to dry for 30-60 min and then the RNA was re-suspended in 30 $\mu\text{l}$  of molecular grade water. The RNA was stored at  $-80^\circ\text{C}$ .

#### 2.4.2. cDNA synthesis

cDNA synthesis converts RNA to DNA template that can be amplified by Taq. All solutions were handled inside the PCR cabinet, and the kit used was the Invitrogen SuperScript II Reverse Transcriptase kit. 1µl of random hexamers (primers) were added to 10µl of each RNA sample. A negative control with water instead of primers and a positive control with water instead of RNA were also set up. The RNA extraction control was also run as a control reaction. The tubes were incubated for 10 min at 70°C. A mastermix was set up for the number of tubes plus one amount of reaction mix. The mastermix for one tube involved 3µl molecular grade water, 4µl 5X Buffer, 2µl 0.1M DTT, 0.6µl 5mM dNTP and 0.4µl Superscript enzyme. Once the 70°C incubation was finished 10µl of mastermix was added to each tube. The tubes were then put into the thermocycler on programme 'cDNA2' which incubated the samples at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min. The samples of cDNA were diluted 1:5 and then stored in the -20°C freezer.

#### 2.4.3. Genomic DNA wipeout cDNA synthesis

All solutions were handled inside the PCR cabinet, and the kit used was Qiagen Quantitect Reverse Transcription kit to remove any traces of gDNA present within the RNA sample. 2µl of gDNA Wipeout Buffer was added to 10µl of each RNA sample, with 2µl of RNase-free water. The tubes were incubated for 5 min at 42°C. A mastermix was set up with the number of tubes plus one amount of reaction mix. The mastermix for one tube involved 4µl 5x Quantiscript Buffer, 1µl RT Primer Mix and 1µl Quantiscript Reverse Transcriptase enzyme. Once the 42°C incubation was finished, the tubes were placed on ice and 6µl of mastermix was added to each tube. A negative control with water instead of enzyme and a positive control with water instead of RNA were also set up (at half volumes). The RNA extraction control was run as a further control reaction. The tubes were then put into the heat block at 42°C for 30 min after which the temperature was increased to

95°C for 5 min. The samples of cDNA were diluted 1:5 and then stored in the -20°C freezer.

#### 2.4.4. Nanodrop

The nanodrop protocol allows the measurement of the concentration of a RNA or DNA solution. 2µl of water was placed onto the nanodrop machine, in order to initiate and then blank the measurement. Either DNA or RNA readings were chosen in the nucleic acid section. Then 2µl of solution was placed and measured. This gives the concentration of the DNA or RNA in ng/µl.

#### 2.4.5. Classical PCR

The HotStarTaq DNA Polymerase kit was used to perform classical PCR to amplify the DNA of interest. The mastermix for this method with or without Q solution is shown in Table 2.2, and the primers used are shown in Table 2.4. 20µl of this mix was placed into each tube, and 5µl of template DNA, or nuclease-free water as a negative control, was added. The tubes were placed in a thermocycler at 94°C for 15 min, then 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 3 min. The last extension step was 10 min at 72°C. The products were then visualised on a 0.8% agarose gel.

#### 2.4.6. Gel electrophoresis

An agarose gel at either 0.8% or 2% was made up (Table 2.5). The gel was left to cool for at least 30 min and then could be placed in the gel tank with gel electrophoresis buffer (Table 2.5). 2µl of BlueJuice loading buffer was added to each sample before loading into the wells of the gel. A marker (2log or 100bp) was loaded in the first well of each lane, 10µl per well. The powerpack was then connected to the gel tank via the lid and set to 120V for 45 min.

Once the gel had run, it was taken from the gel tank and placed in the gel doc machine. The gel was exposed to ultraviolet light for an automated length of time, and this was adjusted if necessary. A picture was taken in this machine and the DNA sample fragment sizes analysed compared to the marker.

#### 2.4.7. Real-Time PCR

Real-time PCR (qRT-PCR) was used to amplify the DNA of the gene of interest and was carried out using qPCR Core kit for SYBR® Green I. The house-keeping gene, HPRT, was used to quantify expression of the target gene using the  $2^{-\Delta C_t}$  method. This procedure was either carried out with Sybr Green as the probe or with a specially made Taqman probe (ABI protocol). For the former the final volume was 25µl, for the latter 20µl. A mastermix with the number of reactions, plus one, was made up in the PCR cabinet. The reagents and quantities are shown below in Table 2.2. The conditions for the different PCR reactions carried out are in Table 2.3. qRT-PCR was performed on an ABI 7500 real-time PCR system.

Reagent	Sybr Green qRT-PCR (μl)	Taqman qRT-PCR for CCK-2R (μl)	Classical PCR (μl)	Classical PCR with Q solution (μl)	LATE-PCR for CCK-2R (μl)
Water	12.63	8.90	15.05	10.05	6.90
10X buffer	2.50	2.00	2.50	2.50	2.00
50nM MgCl	1.75	2.00	-	-	2.00
5mM dNTP	1.00	0.80	0.25	0.25	0.80
Sybr Green	0.75	-	-	-	-
HotGoldStar (enzyme)	0.13	0.10	-	-	0.10
HotStarTaq (enzyme)	-	-	0.20	0.20	-
UNG	0.25	0.20	-	-	0.20
5mM Primers (Table 2.4)	1.00	-	1.00 of each	1.00 of each	-
ABI primer /probe mix (Table 2.4)	-	1.00	-	-	-
LATE-PCR primers					1.00 (F+R together)
LATE-PCR probe					1.00
Q solution	-	-	-	5.00	-
Add to wells	20	15	20	20	15
Template	5	5	5	5	5

Table 2.2: Reagents for real-time and classical PCR

#### 2.4.8. LATE-PCR

This assay is an alternative to qRT-PCR, but modified to make the detection more sensitive. As before the house-keeping gene, HPRT was used to quantify expression of the target gene using the  $2^{-\Delta Ct}$  method [155]. The reagents and conditions are detailed in Table 2.2 and Table 2.3 respectively. The CCK-2R and HPRT probe and primers used were designed specifically for LATE-PCR. There was one limiting primer (reverse primer), at a final concentration of 50nM, and a primer in excess (forward primer), at a final concentration of 1μM. The probe was used at a final concentration of 1μM. The primers (together) and the probe were diluted in molecular grade water to the correct concentration in order to put 1μl of each in the PCR mastermix. Cycling was carried out on an ABI 7500 real-time PCR system, later changed to an ABI StepOnePlus real-time PCR system after similarity testing.

Stage	Step	qRT-PCR (Taqman)		qRT-PCR (Sybr Green)		1st LATE-PCR		2nd LATE-PCR	
		Temp	Time	Temp	Time	Temp	Time	Temp	Time
1		50	2 m	50	2 m	50	2 m	50	2 m
2		95	10 m	95	10 m	95	3 m	95	3 m
3	1	95	15 s	95	15 s	95	10 s	95	10 s
	2	60	1 m	60	1 m	53	10 s	53	10 s
	3					72	20 s		
	4					45	20 s		
4	1							95	10 s
	2							53	10 s
	3							72	20 s
	4							45	20 s
Disso- ciation	1			95	15 s				
	2			60	1 m				
	3			95	15 s				
No. of cycles			40 (st 3)		40 (st 3)		60 (st 3)		10 (st 3) 50 (st 4)

Table 2.3: Real-time PCR conditions, the detection step is shown in green

For the cell-line experiments in Section 3.6 this was further modified to reduce gDNA detection, by elimination of the extension and detection step from the first ten cycles of the PCR (Table 2.3). This method was used for the LATE-PCR results detailed from Section 3.6 onwards.

Method	Primer	Sequence	T <sub>m</sub> (°C)
LATE-PCR	CCK-2R Fc	GGCACATTCATCTTTGGCACCGTCATC	63
	CCK-2R Rc	CGCTCCAGTGCATGGCCACGAGGCTT AGC	68
	HPRT Fc	ACCTAATCATTATGCTGAGGATTTGGAA AGGGTGT	61
	HPRT Rc	GAGGGCTACAATGTGATGGCCTCCCAT CTCCTTCATCAC	70
Taqman qRT-PCR	Lui CCK-2R F	TCATCTTTGGCACCGTCATC	
	Lui CCK-2R R	GATGGCCACGAGGCTTAGC	
	HPRT F	ATTATGCTGAGGATTTGGAAAGGG	
	HPRT R	GCCTCCCATCTCCTTCATCAC	
Sybr Green	HPRT F	ATTATGCTGAGGATTTGGAAAGGG	
	HPRT R	GCCTCCCATCTCCTTCATCAC	
	Gastrin F	CGAGCCTCTCATCATCGAAGG	
	Gastrin R	TTCTTCTTCTTCTCCAGCCATG	
	CD133 4F	AATCTCCCTGTTGGTGATTTG	
	CD133 5R	TCCGGGTTCTTACCTGGTGAT	
	VEGF F	ACGAGGGCCTGGAGTGTGT	
	VEGF R	TTTGTGTGCTGTAGGAAGCTCAT	
CCK-2R promoter	F	TTGACATCCTCTTGACGCTC	
	F1	GAGCAAACAGGGAGCGTTAG	
	F2	CAGCTGAGGGCCATCTTTTA	

	F3	AGAGGAAGCCAGGGGAAATA	
	F4	GGGTGACAGTCATTGGTGGT	
	F5	AATCCCCCATAAAGCCAGAC	
	F6	CAACTGCCCTCAACACTCAA	
	F7	TCTACCCAACCCCTCCTACC	
	F8	ACAGGGAGGGGCAGAACT	
	R	CCGCCTTACTCAGCTCGAC	
	R1	TCCCTGTGCGACTGTCCT	
	R2	CCCCCTCCCTAGATGTGTC	
	R3	AGTTCTGCCCTCCCTGT	
	R4	TTGCAGTTAAGGGTCCCAAG	

**Table 2.4: Primers used in PCR reactions**

#### 2.4.9. Luciferase and $\beta$ -galactosidase assay on the luminometer

The luciferase and  $\beta$ -galactosidase assays for transfection lysates were carried out together so that the second of these normalised the results from the first. Each assay was carried out in triplicate, and therefore 5 $\mu$ l of each lysate was added to three wells in each of two black Nunc luminometer 96-well plates, leaving three wells as blanks. The  $\beta$ -galactosidase reaction mix was made up as a 100-fold dilution of  $\beta$ -galactosidase substrate in the diluent from the Tropix  $\beta$ -galactosidase kit. Immediately, 25 $\mu$ l of this reaction mix was added to each well in the first 96-well plate, the lid was replaced and the plate covered with aluminium foil and left to incubate for 75 min.

The second 96-well plate containing the lysates had 25 $\mu$ l of single reporter (firefly luciferase) substrate added to each well, and the luminometer was run immediately at 1 sec/well. After incubation the  $\beta$ -galactosidase, the (first) 96-well plate was run in the luminometer at 1 sec/well. The mean was taken of the triplicate values for luciferase and  $\beta$ -galactosidase separately, and then the luciferase expression mean was divided by the  $\beta$ -galactosidase expression mean in order to achieve normalised (to account for different living cell number) results for luciferase expression.



#### 2.4.10. Western blotting

This was a preliminary experiment only and was optimised by previous laboratory users. The cells were harvested using the Biovision nuclear/cytosolic fraction kit. The western blotting kit used was Novex from Invitrogen and the primary antibody was mouse anti-human HIF-1 $\alpha$  (BD Biosciences) at 1:250, with a secondary biotinylated antibody and the ABComplex/Strept and ECL kits were used for development and visualisation of bands.

### 2.5. Bacteria and cloning

#### 2.5.1. TOPO cloning

This method was used to ligate classical PCR products into the TOPO plasmid, in order that they could then be moved into pGL4. The kit for this protocol was the TOPO TA Cloning<sup>®</sup> Kit. For each reaction, 1 $\mu$ l of classical PCR product, 1 $\mu$ l of salt solution, 1 $\mu$ l of pCR<sup>®</sup>II-TOPO<sup>®</sup> plasmid (Figure 2.1) and 3 $\mu$ l of molecular grade water were mixed together and incubated for 5 min at room temperature. This could then be transformed into One Shot<sup>®</sup> TOP10F' chemically competent cells.

#### 2.5.2. Plasmids used

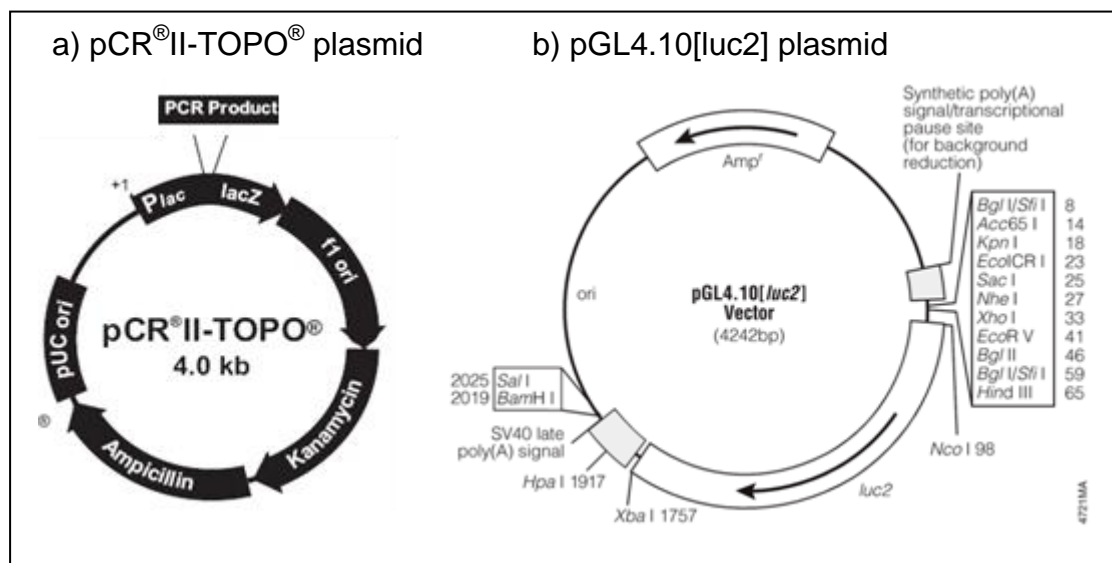


Figure 2.1: Plasmid vectors used in this project

### 2.5.3. Transformation of chemically competent cells

2µl of the solution from TOPO cloning was added to a vial of One Shot® TOP10F' competent E. coli, mixed gently and incubated on ice for 5-30 min. The cells were then heat-shocked at 42°C for 30 sec, and immediately transferred back to the ice. 250µl of warmed SOC medium was added to each vial and these were put into a universal and shaken at 200rpm in a 37°C incubator for 1 hour. 10-50µl of this mixture was spread onto previously-prepared LB-Amp plates with X-gal/IPTG. The plates were placed in a 37°C incubator overnight and the blue colonies were picked for bacterial culture 24 hours later.

### 2.5.4. Digest (for test or purification)

DNA digestion was used to remove the promoter fragments from the TOPO plasmid and to linearise pGL4 with the same enzymes, to ensure a simple ligation of these molecules. A sample volume of 10µl was used for most restriction endonuclease (RE) digests, except for those leading to gel extraction. This volume consisted of 1µl of test DNA, 1µl of NEB 4 buffer, 0.5µl of each RE, 1µl of BSA if necessary and the rest made up with nuclease-free water. This solution minus the DNA was made up as a mastermix and then each tube had 9µl of this and 1µl of DNA added. The tubes were put into the incubator at 37°C for 90 min. The digest solution was run in gel electrophoresis to separate the fragments produced.

### 2.5.5. DNA gel extraction

The process of making promoter plasmids included removing fragments of DNA from TOPO plasmids and annealing them into the pGL4 plasmid, and gel extraction was used to ensure only the correct fragments entered this ligation. Once the gel had separated the DNA fragments sufficiently that they could be confidently cut apart, and a picture taken, the gel was transferred to the UV bed. Each fragment

was carefully cut out of the gel with a clean scalpel blade and placed in a labelled eppendorf. The other fragments in the gel were not touched in order to keep the fragment as clean as possible. Each gel fragment was weighed compared to an empty eppendorf and the weight noted on the tube.

The gel extraction protocol is taken from the Qiagen QIAquick Gel Extraction Kit. Each centrifugation was carried out at maximum speed using a benchtop centrifuge. Three gel volumes (100mg≈100µl) of QG buffer were added to the eppendorf, and heated to 50°C for 10 min. Once the gel was dissolved, one gel volume of isopropanol was added to each tube and mixed together. This solution was added to a QIAquick column in 750µl aliquots, centrifuging for one min then removing the eluate after each addition. 500µl of QG buffer was added to the column and spun for one min, before 750µl of PE wash buffer was added and again the column was centrifuged for 1 min. The eluate was discarded and the column spun again for one min to dry the filter. This column was then transferred to a new collection tube and 30µl nuclease-free water was added to the filter and the tube left to stand for one min. Centrifuging for one min produced the pure DNA fragment and this could be ethanol precipitated or stored at -20°C.

#### 2.5.6. Ethanol precipitation

The DNA used for ligation and transfection needed to be pure, and ethanol precipitation was used to ensure this. The DNA solution was measured and 1/10th the volume of 3M NaAc was added to it. 2x the new total volume of 100% ethanol was added to this and the tube was placed at -20°C for one hour/overnight. The tubes were then centrifuged at maximum speed for 15 min at 4°C and the supernatant poured away. 1ml of 70% ethanol was added to wash and the tubes centrifuged again at 4°C for 5 min, before pouring off all the wash solution. The pellets

were dried under tissue paper for 30 min and then re-suspended in enough nuclease-free water to make up the correct concentration (the DNA concentration was determined using the nanodrop before starting).

#### 2.5.7. Ligation

This method ligated the DNA fragments cut from the TOPO plasmid and the linearised pGL4 vector. 100ng of vector (pGL4; Figure 2.1) and a 1:3 ratio of insert DNA were used in each positive ligation, and the negative for each sample had vector alone. This meant that the volume of insert needed was  $3 \times 100 \times (\text{insert bp} / \text{vector bp})$ . If the volume of this mixture exceeded 7µl then they were mixed together and ethanol precipitated to make them up to 7µl. 1µl each of ligation buffer, ATP and ligase were then added. If the total volume did not make 10µl then nuclease-free water was added. The tubes were then left overnight at 4°C.

#### 2.5.8. Transformation of competent/supercompetent cells

In order to produce enough of the ligated plasmid for transfection this was transformed into fast-growing *Escherichia coli* cells. 50µl of XL1-Blue competent or supercompetent cells per sample were defrosted on ice. These were then transferred to a cooled 15ml falcon tube, 0.85µl of β-mercaptoethanol was added and the tube swirled carefully. These were incubated on ice for 10 min with swirling every 2 min, and then 50ng of the DNA or 1µl ligation mix was added. This was again incubated on ice for 30 min, while heating a water bath to 42°C precisely. The cells were heat-shocked at 42°C for 45 sec before ice incubation for 2 min. 450µl of warmed SOC medium was added to the falcon tube and then it was left at 37°C with shaking at 230rpm for 1 hour.

The cells were dropped and spread onto previously-prepared LB-Amp agar plates at a suitable volume. This was a small amount (10-50µl) for certain DNA transformations and all of the cells on two plates for ligation transformations. The negative control transformations (from negative ligations) were plated only on one plate. Once dry, the plates were placed upside-down in the 37°C incubator overnight, and the colonies examined in the morning. If the plates were covered with colonies then these were re-streaked onto fresh plates and left overnight again.

#### 2.5.9. Bacterial culture

For each culture, 2 or 5ml of LB-Amp was made up as a mastermix, and then aliquoted into universals. The bacterial stocks were defrosted on ice, if necessary. A new disposable loop was taken for each culture, and dipped in the bacterial stock eppendorf or touched to a bacterial colony from an agar plate. This was then mixed in the universal containing LB-Amp and disposed of in 10% Trigene. The cultures were put into the 37°C incubator, with shaking at 230rpm, overnight.

#### 2.5.10. Bacterial stocks

0.5ml of each culture was taken into an eppendorf and 0.5ml of LB-glycerol was added. This was mixed and the labelled tube of bacteria frozen down at -70°C.

#### 2.5.11. Plasmid mini-prep

The plasmid DNA was removed from *E. coli* for use in transfections. The bacterial cells were harvested from the overnight cultures into 1.5ml eppendorfs by centrifugation at 13,000rpm for 3 min. The plasmids from these bacteria were isolated using the Sigma Genelute Plasmid Mini-prep Kit, and all centrifugation was carried out at

maximum speed. The pellet was re-suspended in 200µl re-suspension solution, and then 200µl lysis solution was added and the tube mixed. The solution was left to clear for 5 min and then 350µl of neutralisation solution was added and the tube inverted 6 times. This was centrifuged for 10 min, and the supernatant from this was transferred to a spin column, which had been previously centrifuged for 1 min with 500µl preparation solution added.

The spin column was centrifuged for 1 min, and then 750µl wash buffer added, before being centrifuged for one min again. The eluate was removed and the column dried with another spin for 1 min, before the column was transferred to a new collection tube and 100µl of nuclease-free water was applied to the centre of the filter. Centrifugation for one min produced the purified plasmid DNA and the tube was labelled and frozen at -20°C. The DNA concentration was often tested in the nanodrop before ethanol precipitation or freezing, and adjusted to 100ng/µl if it was to be used for transfection.

## 2.6. Table of media

Medium and Reagents	Recipe
0.8% Agarose gel	0.8g agarose powder and 100ml 1x TAE. Heated until dissolved. 5µl Safeview added, the mixture poured into a tray, and the comb placed in. Left to cool.
2% Agarose gel	As above with 2g of agarose powder.
70% Ethanol	35ml 100% ethanol with 15ml molecular grade water.
Freezing medium	Normal growth medium with 10% DMSO.
Gel electrophoresis buffer	600ml 1x TAE and 30µl Safeview.
HEPES buffer	RPMI 1640 medium with 2% Foetal Bovine Serum (FBS) and 5mM HEPES.
LoVo medium	F-12 Ham medium with 10% FBS and 2mM glutamine.
Luria-Bertani medium with ampicillin (LB-amp)	5g NaCl, 2g MgSO <sub>4</sub> , 5g yeast extract and 10g casein hydrosylate added to a beaker and filled up to 900ml with distilled water. pH to 7.5 and autoclaved before use.
LB-Ampicillin Agar plates	One sachet of FastMedia LB Agar Amp poured into 200ml autoclaved distilled water, heated until dissolved. Poured into

	petri dishes when cooler.
LB-Ampicillin Agar plates with X-gal and IPTG	As above, but when set, 40µl of 100mM IPTG was spread on the plate, and then 40µl of 40mg/ml X-gal was spread on top.
LB-glycerol	Autoclaved LB medium with 15% glycerol added. Filtered before use.
Normal growth medium	RPMI 1640 medium with 10% FBS and 2mM glutamine.
Phosphate buffered saline (PBS)	1 tablet of PBS dissolved in every 100ml distilled water. Filtered before use in tissue culture.
Serum free medium	Normal growth medium, minus the FBS.
Stem Cell Medium (SCM)	372 ml Low Glucose DMEM plus 2.5ml heparin, 116ml F-12 Ham, 10ml B27 supplement and (added fresh) 20µg/ml EGF and FGF.
1x Tris Acetate EDTA (TAE)	100ml 10x TAE stock and 900ml distilled water.

**Table 2.5: Medium and reagents used in this project**

## 2.7. Table of materials

Material	Cat. number	Supplier
100bp DNA ladder	N3231L	New England Biolabs
2log DNA ladder	N3200L	New England Biolabs
ABC kit	AK-5003	Vector Laboratories
AEC kit	SK-4200	Vector Laboratories
Agarose powder	EP-0010-05	Eurogentec
Ampicillin	A5354	Sigma Aldrich
B27	17504-044	Invitrogen
Biotinylated antibody rabbit α-goat	E0466	Dako
Biotinylated antibody swine α-rabbit	E0353	Dako
Bluejuice loading buffer	10816-015	Invitrogen
Bovine serum albumin (BSA)	B90015	New England Biolabs
Casein hydrosylate	22090	Fluka
CCK-2R primary antibody	ab14440	Abcam
CD133 primary antibody	130-092-395	Milteni
CD44 conjugated primary antibody	560532	BD Biosciences
Chloroform	C-2432	Sigma Aldrich
Citifluor	AF2	Science Services
Citric acid	C1857	Sigma Aldrich
DAB kit	K4065	Dako
Deoxyribonucleotide triphosphates (dNTPs)	DNTP-100A-1KT	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	D4540	Sigma Aldrich
DMEM (low Glucose)	D6046	Sigma Aldrich
DPX	44581-100ML	Sigma Aldrich
Epidermal growth factor (EGF)	13247-051	Invitrogen
Ethanol	E7023	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	E6758	Sigma Aldrich
Exonuclease III	M1811	Promega

F-12 Ham medium	21765	Gibco
FastMedia LB Agar Amp	#M0021	Fermentas
Fibroblast growth factor (FGF)	PHG 0026	Biosource
Firefly luciferase substrate	E1483	Promega
Foetal Bovine Serum (FBS)	F7524	Sigma Aldrich
Formaldehyde	F8775	Sigma Aldrich
Genelute plasmid Miniprep kit	PLN350	Sigma Aldrich
Geneticin	A1720	Sigma Aldrich
L-Glutamine	G8540	Sigma Aldrich
Glycerol	G-5516	Sigma Aldrich
Goat $\alpha$ -mouse secondary FITC-labelled antibody (AlexaFluor 488)	A11001	Invitrogen
Goat $\alpha$ -rabbit secondary FITC-labelled antibody (AlexaFluor 488)	A11008	Invitrogen
Goat Serum	G9023	Sigma Aldrich
Haemalum	51275-100ML	Sigma Aldrich
Heparin	H1027	Sigma Aldrich
HEPES	H4034	Sigma Aldrich
HIF 1 $\alpha$ and HIF 2 $\alpha$ siRNAs	-	Eurogentec [156]
Hoechst	H21486	Invitrogen
HotStarTaq DNA Polymerase	203203	Qiagen
Hydrogen peroxidase	323381-500ML	Sigma Aldrich
Isopropanol	I9516	Sigma Aldrich
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	I1284	Sigma Aldrich
Isotype conjugated control antibody	558331	BD Biosciences
Klenow DNA polymerase	M2201	Promega
Ligase, 10x Ligation buffer, ATP	600011 (T4 kit)	Stratagene
Lipofectamine 2000	11668-019	Invitrogen
Methanol	650188	Sigma Aldrich
MgSO <sub>4</sub>	M/1050/53	Fisher Scientific
Molecular grade water	W4502-1L	Sigma Aldrich
Mouse serum	M5905	Sigma Aldrich
NaCl	S/3160/63	Fisher Scientific
NEBuffer 4	B70045	New England Biolabs
OptiMEM1 medium	31985-047	Invitrogen
PBS tablets	BR0014G	Oxoid
Plasmid: pGL4		
Plasmid: TA GAS I1 1.1, 1.2, 1.3	In house	-
Polyethylene glycol	76293	Sigma Aldrich
Primers and probes for Taqman qRT-PCR and LATE-PCR	-	Eurogentec
Prolong® Gold	P36930	Invitrogen
QIAquick gel extraction kit	28704	Qiagen
Quantitect Reverse Transcription kit	205311	Qiagen
qPCR Core kit for SYBR® Green I	RT-SN10-05	Eurogentec
Rabbit serum	R9133	Sigma Aldrich
Random hexamers	N8080127	Invitrogen
Restriction Endonuclease (RE): NcoI, XhoI, XmaI	R01935, R01465, R01805	New England Biolabs
Restriction Endonuclease (RE): SacI	R606E	Promega



RPMI 1640 medium	R0883	Sigma Aldrich
S1 nuclease	M5761	Promega
Safeview nucleic acid stain	NBS-SV1	NBS Biologicals
Silencer siRNA construction kit	AM1620	Ambion
siPORT Amine	AM4503	Ambion
siRNA: gastrin	-	Eurogentec
siRNA: negative	SR-CL000-005	Eurogentec
siRNA: scrambled gastrin	-	Eurogentec
siRNA: scrambled gastrin TAMRA-labelled	-	Eurogentec
SOC medium	S1797	Sigma Aldrich
SuperScript II Reverse Transcriptase kit	18064-022	Invitrogen
Swine serum	sc-2486	Santa Cruz Biotech
10x TAE	T9650	Sigma Aldrich
TOPO TA Cloning® Kit	K4650-01	Invitrogen
TRI reagent	T9424	Sigma Aldrich
Trigene	TM308	Medichem International
Trypan blue dye	T6146	Sigma Aldrich
Trypsin-EDTA	T3924	Sigma Aldrich
Universal negative control for mouse	IS750	Dako
Uracil N-glycosylase (UNG)	RT-0610-15	Eurogentec
VectaMount	H-5501	Vector Laboratories
X-gal (5-Bromo-4-chloro-3- indolyl $\beta$ -D-galactopyranoside)	B6024	Sigma Aldrich
XL1-Blue competent/supercompetent cells	200249/200236	Stratagene
Yeast extract	Y-1625	Sigma Aldrich

**Table 2.6: Materials used in this project**

## Chapter 3    Detection of the CCK-2R

### 3.1. CCK-2R expression

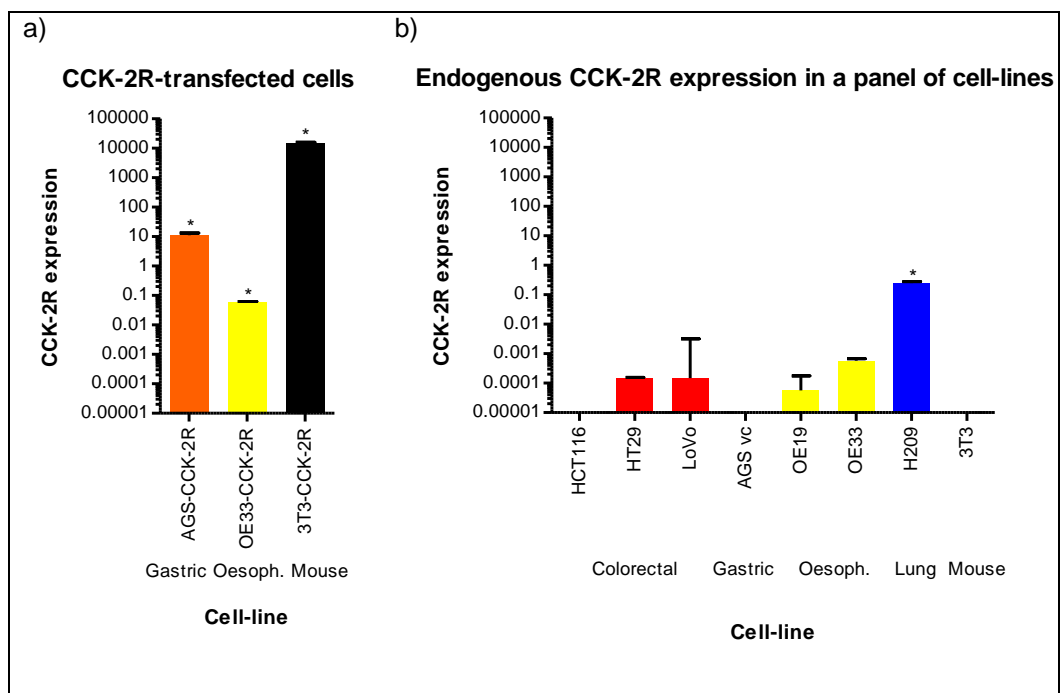
Reported levels of CCK-2R gene and protein expression in cancer cells vary widely in the literature [72]. The aim of this chapter was to investigate CCK-2R gene expression in a panel of cell-lines, using a new method for real-time PCR, and to validate this technique using RNAi. Protein expression was also investigated and correlated with gene expression.

### 3.2. CCK-2R expression in a panel of cell-lines

CCK-2R expression was initially investigated in a panel of cancer cell-lines including gastric, colorectal, pancreatic, oesophageal and SCLC cells using a Taqman probe in qRT-PCR. The primers used have been previously published by Liu *et al.* [157]. Several controls were devised to assess the assay; in-house CCK-2R-transfected cell-lines were used as positive controls, and NIH3T3 mouse fibroblast cells were used as a negative control, since there is low homology between the primers used and the mouse CCK-2R gene. Together these provide an important measure of the sensitivity of the assay.

A cDNA negative control was included in the qRT-PCR for each cell-line. This consisted of the cDNA synthesis sample with RNA but no primers or enzyme, and therefore should not have contained any DNA. This control must show a Ct value of at least 10 cycles higher than that of the equivalent positive cDNA sample, to confirm that genomic DNA (gDNA) contamination is not present. This is a particular problem for detection of CCK-2R due to the primers being designed over intron 2, which is only 167bp long. The amplicon itself is 86bp, illustrating that the reaction could amplify this together with the intron without a long extension time, if gDNA is present in the cDNA sample. Unfortunately the samples from MGLVA-1 (gastric) and PAN-1 (pancreatic) cells had this contamination and therefore are not shown in Figure 3.1.

Three human CCK-2R-transfected cell-lines (Figure 3.1a) were tested along with their vector controls (VCs), or if these were not available, wild-type (wt) cells. The transfected lines showed the highest expression levels, as expected, and all three showed at least 10,000-fold more expression of the receptor than their VC/wt ( $p < 0.05$ ). This indicates that the assay detects CCK-2R mRNA. The negative control used; the wild-type mouse fibroblast cell-line, NIH3T3, did not show any expression of human CCK-2R, showing the specificity of the Taqman qRT-PCR. Figure 3.1 shows these results, using the  $2^{-\Delta C_t}$  method [155] which provides a measure of the expression of CCK-2R relative to the housekeeping gene, HPRT [158].



**Figure 3.1: Expression of CCK-2R by Taqman qRT-PCR**

Logarithmic graphs showing the expression of CCK-2R ( $2^{-\Delta C_t}$ ) relative to the housekeeping gene, HPRT, in a panel of cell-lines, using Taqman qRT-PCR. CCK-2R-transfected cell-lines are shown in a), while b) shows endogenous expression in wild-type cells. For each cell-line, data was obtained for three cDNA replicates. The black bars represent the 95% confidence interval, and a \* above the bar indicates that it is significantly different ( $p < 0.05$ ) to all other bars (on both graphs, except OE33-CCK-2R and H209 which are not statistically different in terms of CCK-2R expression).

Five wild-type cell-lines showed endogenous CCK-2R expression; HT29 and LoVo (colorectal), OE19 and OE33 (oesophageal) and H209 (SCLC). SCLC cells were included in the study since they have been

previously shown to express CCK-2R by our and other groups, by use of classical RT-PCR [159]. Except for H209 cells, endogenous expression of the CCK-2R seems to be 100-fold lower than in transfected OE33-CCK-2R cells. Compared to NIH3T3-CCK-2R cells, the level of the endogenous receptor gene in H209 cells is 10,000-fold lower.

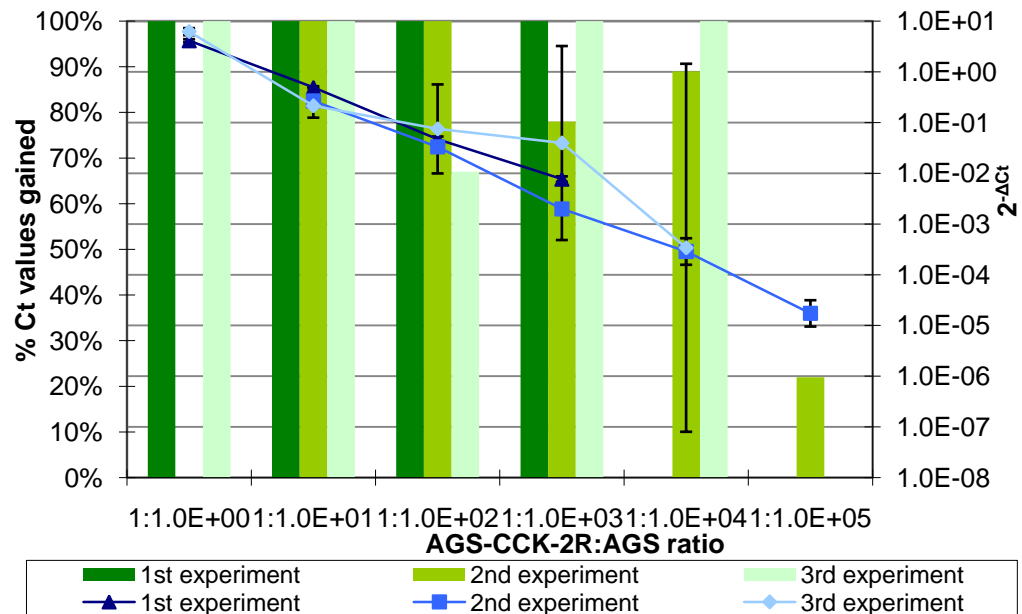
However, HCT116 (colorectal) and AGS vector control (gastric) cells did not show any expression despite the findings of several inhibitor studies which suggest that the receptor is present [74, 139]. This result suggests that Taqman qRT-PCR is not sensitive enough to detect the level of CCK-2R gene activity occurring in these cell-lines.

### **3.3. CCK-2R Taqman assay sensitivity studies**

In order to test the sensitivity of the CCK-2R Taqman assay formally, RNA was prepared from a series of dilutions of AGS-CCK-2R cells in untransfected CCK-2R-negative AGS cells, so that the concentration of total RNA in the samples remained the same throughout, while the CCK-2R RNA was titrated out. Figure 3.2 shows that the Taqman qRT-PCR could consistently detect signal when the CCK-2R was diluted to 1:1.0E+02. At 1:1.0E+03, however, the results become less uniform, as can be seen by the increased size of the confidence interval at this ratio and beyond. By the 1:1.0E+06 dilution CCK-2R expression could not be detected.

The proportion of wells where a Ct value was detected is also indicated in Figure 3.2. Beyond the 1:1.0E+02 dilution some Ct values were classified 'undetectable' by the ABI software. Therefore, a value of 40 was used as the Taqman qRT-PCR was run to 40 cycles, but the Ct values may be larger than this. This indicates that expression of the CCK-2R gene is at the limit of detection of the assay. The proportion of

positive wells (with a Ct <40) decreases with increasing dilution as predicted by the Poisson distribution. For example, in the bar chart within Figure 3.2, it can be seen that with a 1:1.0E+05 dilution of the CCK-2R, the receptor was detectable in a total of 2 out of 12 samples (it was not detected at all in the third experiment).



**Figure 3.2: CCK-2R Taqman sensitivity studies**

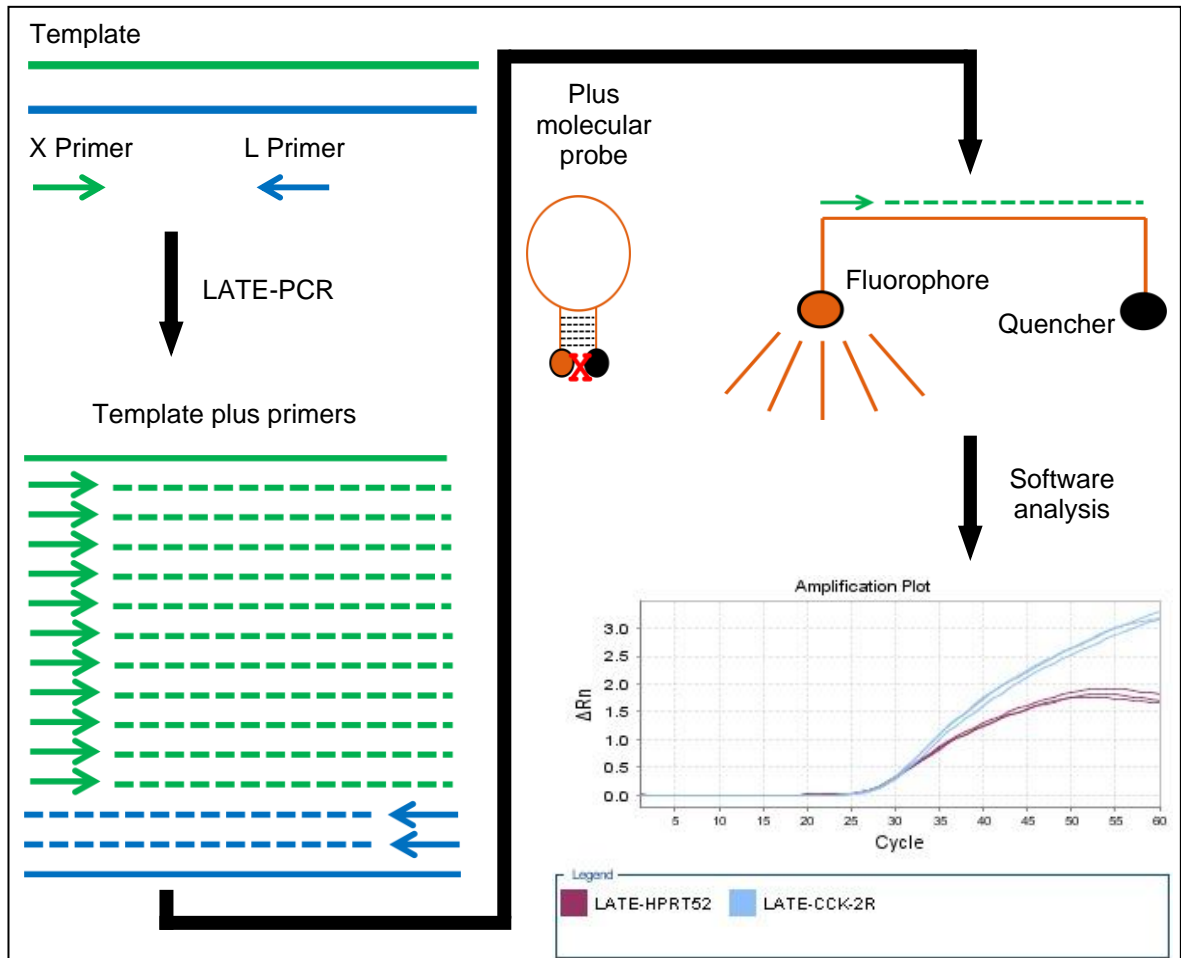
AGS-CCK-2R cells were diluted in AGS cells to produce the dilution series. The experiment was repeated three times, but not all of the ratios were tested each time. The bar chart illustrates the % of wells in which the Ct value was <40. The line graph with error bars demonstrates the expression of CCK-2R relative to HPRT ( $2^{-\Delta C_t}$ ) on a logarithmic scale. The error bars represent the 95% confidence interval.

The 1:1.0E+05 dilution did not yield any results at all in experiment 2 and 3 (Figure 3.2). The differences that occur beyond the 1:1.0E+02 dilution suggest that this is the limit of detection in the assay, and that perhaps a more sensitive assay should be established.

### 3.4. The LATE-PCR assay

A Linear-after-the-exponential (LATE-) PCR was developed to increase the sensitivity of qRT-PCR. This is extremely relevant to detection of CCK-2R mRNA expression since it seems to be present in very low concentrations even in cells where it has been shown to have a

biological effect [13, 74, 138, 139]. LATE-PCR uses different concentrations of each primer to yield primarily single-stranded product (Figure 3.3). This means that the specially designed single-stranded probe can bind easily, therefore reducing the amount of DNA needed before a signal is detected.



**Figure 3.3: The steps of LATE-PCR**

Two primers, at different concentrations, are added to the sample of cDNA being tested for gene expression. These are the limiting (L) primer at a concentration of 50nM, which will be depleted quickly and an excess (X) primer at a concentration of 1μM, to carry on the reaction. The process of LATE-PCR briefly produces a double-stranded product, then when the L primer is depleted, single-stranded product is formed. The specially designed single-stranded molecular probe binds to this product easily, causing the separation of the quencher from the fluorophore. The fluorophore fluoresces allowing the PCR machine to detect the signal, and software analysis shows the PCR cycle at which this first occurs as the Ct value.

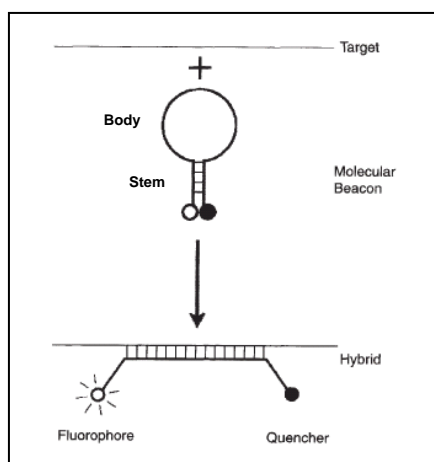
### 3.4.1. Primer design for LATE-PCR

In LATE-PCR, one primer is at a limiting (L), and one at an excess (X), concentration. Their melting temperatures ( $T_m$ ) are important and must obey the rule  $T_m^L - T_m^X \geq 0$  to ensure that the reaction is efficient [160]. The amplicon for this assay was generated from the qRT-PCR CCK-2R (Taqman) and HPRT (Sybr Green, since the extended HPRT Taqman primers and probe did not show any detection) primers used above [157]. The  $T_m$  of the amplicon was calculated using the nearest-neighbour formula detailed in Pierce *et al.* [142], as were those of the primers at a limiting or excess concentration (since this changes the  $T_m$ ). It was recommended that the primer with the highest  $T_m$  at a limiting concentration should be allocated as the limiting primer; in both cases this was the reverse primer. This  $T_m$  needed to be close to that of the amplicon, which involved increasing the 5' end of each primer significantly in order to increase the primer  $T_m$ .

### 3.4.2. Design of the molecular probe

Two probes were designed to complement the product formed using the excess primer. Two types of probe were tested based on two different designs published by the group that first developed LATE-PCR. Both were investigated because LATE-PCR is a new technique and has only previously been applied to a very limited set of genes. A relatively high  $T_m$  probe is necessary in qRT-PCR for quick annealing with the product, while it is single-stranded; however, during LATE-PCR, since the product is almost exclusively single-stranded, a low  $T_m$  probe could also perform. In Sanchez *et al.* [160] they explain why the low  $T_m$  probe should be the more effective of the two since it would bind to the product slowly and therefore be more specific, create lower background and not reduce the efficiency of the reaction.





**Figure 3.4: The mechanism of action of a molecular beacon**

*When the molecular beacon (probe) binds to a sequence the fluorophore and quencher are forced apart allowing the fluorophore to fluoresce. This fluorescence is detected by the real-time PCR machine at a certain cycle number, which gives each sample a 'Ct value'. [161].*

One probe was based on the papers by Pierce *et al.* [142, 162, 163] with a stem of length 6bp (Figure 3.4) and body  $T_m$  between 48°C and 52°C; the second design was based on Sanchez *et al.* [160], and this probe had a shorter stem structure of 5bp in length, but a similar body  $T_m$ . The first of these will be referred to as the 'high  $T_m$ ' probe, since the entire probe  $T_m$  is high (~70°C) and the second as the 'low  $T_m$ ' probe since its entire  $T_m$  is around 67°C ( $T_m$  calculations by 'Melting' programme in [164]). The activity of the high  $T_m$  and low  $T_m$  probes, for the CCK-2R, are compared below.

### 3.4.3. Optimisation of the primers (and probe)

Three forward and reverse primers, of increasing length, were chosen for each target, due to their predicted interaction; Pierce *et al.* [142] found that  $T_m^L - T_m^X \geq 5$  seemed to give high efficiency to their LATE-PCR, therefore our primer pairs were chosen to obey this rule. However, in order to investigate this in LATE-PCR for the genes CCK-2R and HPRT, all nine combinations of the primers for each target were analysed for activity using Sybr Green qRT-PCR, on AGS-CCK-2R cDNA to give a consistent level of gene activity for detection.

Ct values obtained are shown in Table 3.1. The pair which produced the lowest Ct values was chosen, since this meant the earliest detection of the PCR product. For CCK-2R, FcRc was the chosen combination since

this produced a low Ct value, an undetectable negative control and had the desired T<sub>m</sub> ratio (Table 3.1).

Sybr Green qRT-PCR for HPRT produced very similar results for all the primer pairs so the probe was introduced in the optimisation process. This led to the FcRc pair being chosen since they showed the lowest Ct value. Interestingly the selected pair for both CCK-2R and HPRT was that predicted to be the best, being the longest primers of their set, with the highest T<sub>m</sub>'s. This also meant that the T<sub>m</sub><sup>L</sup>–T<sub>m</sub><sup>X</sup> ≥ 5 rule was true for both targets: CCK-2R T<sub>m</sub><sup>L</sup>–T<sub>m</sub><sup>X</sup> = 5 and HPRT T<sub>m</sub><sup>L</sup>–T<sub>m</sub><sup>X</sup> = 9 (Table 3.2).

F primer	R primer	CCK-2R (Sybr Ct)	CCK-2R (negative control)	HPRT (Sybr Ct)	HPRT (negative control)	HPRT (Probe Ct)
Fa	Ra	U	U	24	U	39
Fa	Rb	19	U	24	U	36
Fa	Rc	19	U	23	U	37
Fb	Ra	32 (1/3 U)	U	24	U	32
Fb	Rb	19	28	24	U	31
Fb	Rc	19	32	23	U	33
Fc	Ra	31-34	31	not done	not done	not done
Fc	Rb	19	31	25	U	30
Fc	Rc	19	U	25	U	30

**Table 3.1: Optimisation of the primers for LATE-PCR**

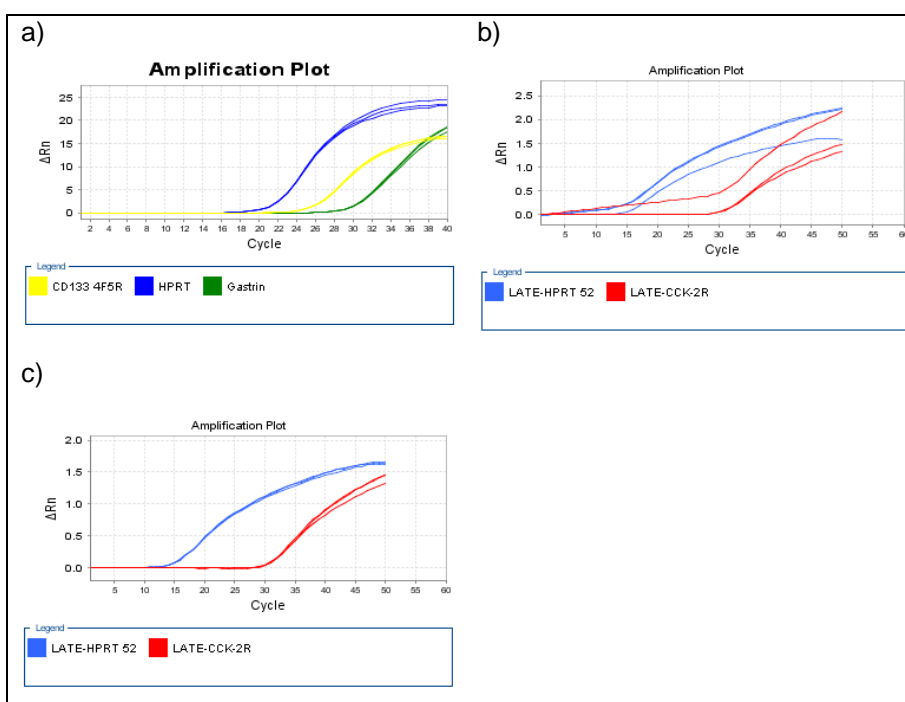
*U is undetectable.*

Primer	Concentration	Sequence	T <sub>m</sub> (°C)
CCK-2R Fc	Excess	GGCACATTCATCTTTGGCACCGTCA TC	63
CCK-2R Rc	Limiting	CGCTCCAGTGCGATGGCCACGAGG CTTAGC	68
HPRT Fc	Excess	ACCTAATCATTATGCTGAGGATTTG GAAAGGGTGT	61
HPRT Rc	Limiting	GAGGGCTACAATGTGATGGCCTCC CATCTCCTTCATCAC	70

**Table 3.2: The primers chosen for use in CCK-2R and HPRT LATE-PCR**

The LATE-PCR assay was run using the chosen primer pairs and the specially designed molecular probes. The amplification curves for this

assay did not look exactly as did those for standard qRT-PCR; these were more S-shaped and with a plateau at the top (Figure 3.5a). Figure 3.5b shows what the amplification plots for LATE-PCR first looked like: they did not always run along the x-axis before the exponential phase. Sanchez *et al.* [160] show several figures explaining that the concentration and ratio of primers are very important to the appearance of the graph in LATE-PCR; so it was possible that further optimisation was necessary.



**Figure 3.5: Amplification plots in qRT-PCR**

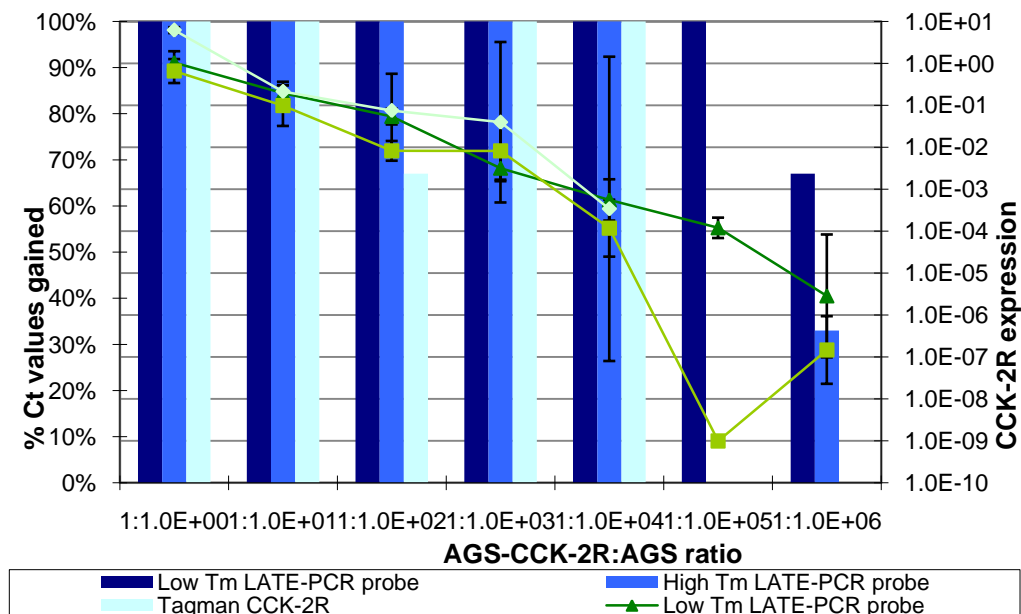
a) Gastrin, CD133 and HPRT Sybr Green qRT-PCR, and b) & c) CCK-2R and HPRT LATE-PCR on a sample of HT29 cDNA. The graph in b) has been plotted with the automatic baseline (3-15), whereas in c) this has been changed to 1-10 cycles; this causes the software to ignore the data which was acquired while the limiting primer was still causing double-stranded product to form. The comparison of the amplification plots shows that CCK-2R LATE-PCR produces a different shape of graph.

Primer and probe concentrations were tested, since those used for the Sybr Green testing had been taken from the literature [142]. The concentrations used in Pierce *et al.* [142] were, the X primer at 1 $\mu$ M, the L primer at 50nM and the probe at 1 $\mu$ M. However, for both assays, these primer concentrations were found to give the best amplification curves, and it was found that changing the baseline of the graphs in the software caused the data to form the expected S-shaped amplification

plot (Figure 3.5c). Despite the optimisation, the Ct values for CCK-2R LATE-PCR were far higher (up to 15 cycles higher) than those for HPRT, which reflected the very low gene expression compared to that of HPRT.

### 3.5. Determining the sensitivity of the LATE-PCR assay

The LATE-PCR was repeated on the same samples used for the Taqman assay, in order to determine its sensitivity. This new assay was run for 60 cycles, since this longer run (compared to the Taqman 40-cycle run) was recommended by Pierce *et al.* [142]. The Taqman assay shown in Figure 3.6 was also run for 60 cycles in order to compare them precisely.

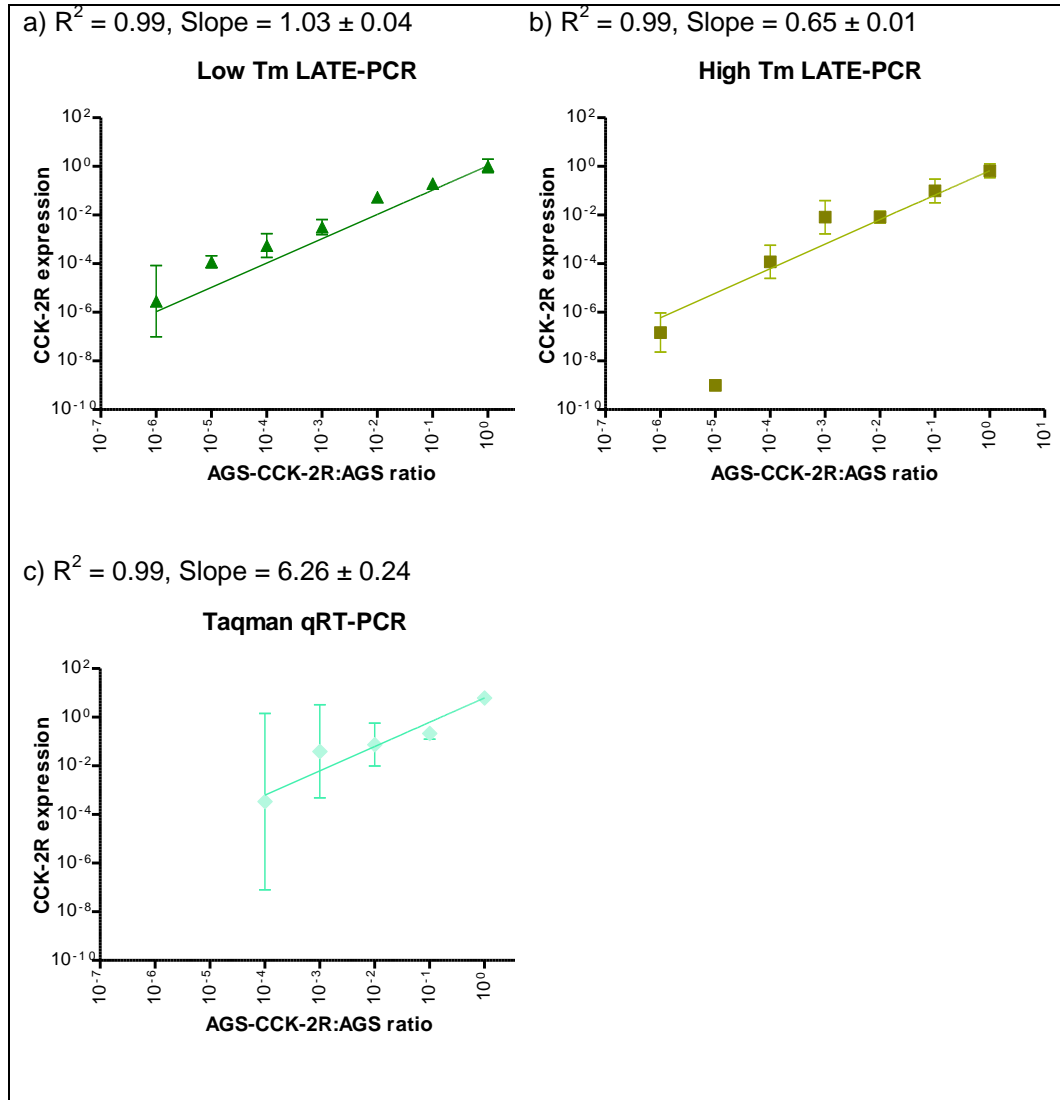


**Figure 3.6: Comparison between LATE-PCR and the CCK-2R Taqman assay**

Two different LATE-PCR probes were used: low Tm (based on Sanchez *et al.* [160]) and high Tm (based on Pierce *et al.* [142]). AGS-CCK-2R cells were diluted in AGS cells in series to produce the ratio samples. The bar chart illustrates the % of wells with Ct values of < 60. The line graph shows the expression of CCK-2R relative to HPRT ( $2^{-\Delta C_t}$ ) on a logarithmic scale. The error bars represent the 95% confidence interval.

The LATE-PCR method is able to detect the CCK-2R at lower concentrations than the Taqman qRT-PCR (Figure 3.6). Both probes for

LATE-PCR detected the receptor at a dilution of 1:1.0E+04, whereas the results for the Taqman assay produced far bigger error bars at this concentration.



**Figure 3.7: Linear correlations performed on the CCK-2R expression data from Figure 3.6**

The  $R^2$  value and slope are described above each graph for a) low Tm LATE-PCR, b) high Tm LATE-PCR and c) Taqman qRT-PCR. The error bars represent the 95% confidence intervals for the PCR data.

The low Tm LATE probe detected the CCK-2R most consistently at the two lowest concentrations (Figure 3.6), with the smallest confidence intervals, suggesting this probe is more sensitive than the high Tm probe. The CCK-2R expression data were used to perform a linear correlation with the concentration of CCK-2R cDNA present in the samples (Figure 3.7). The linear correlation shows that although all

three methods have  $R^2$  values of 0.99, their slopes are very different. The low  $T_m$  probe produces a line with a slope of 1, indicating a ten-fold change in signal for a ten-fold change in dilution, whereas this was not the case when the high  $T_m$  and Taqman probes were used (Figure 3.7).

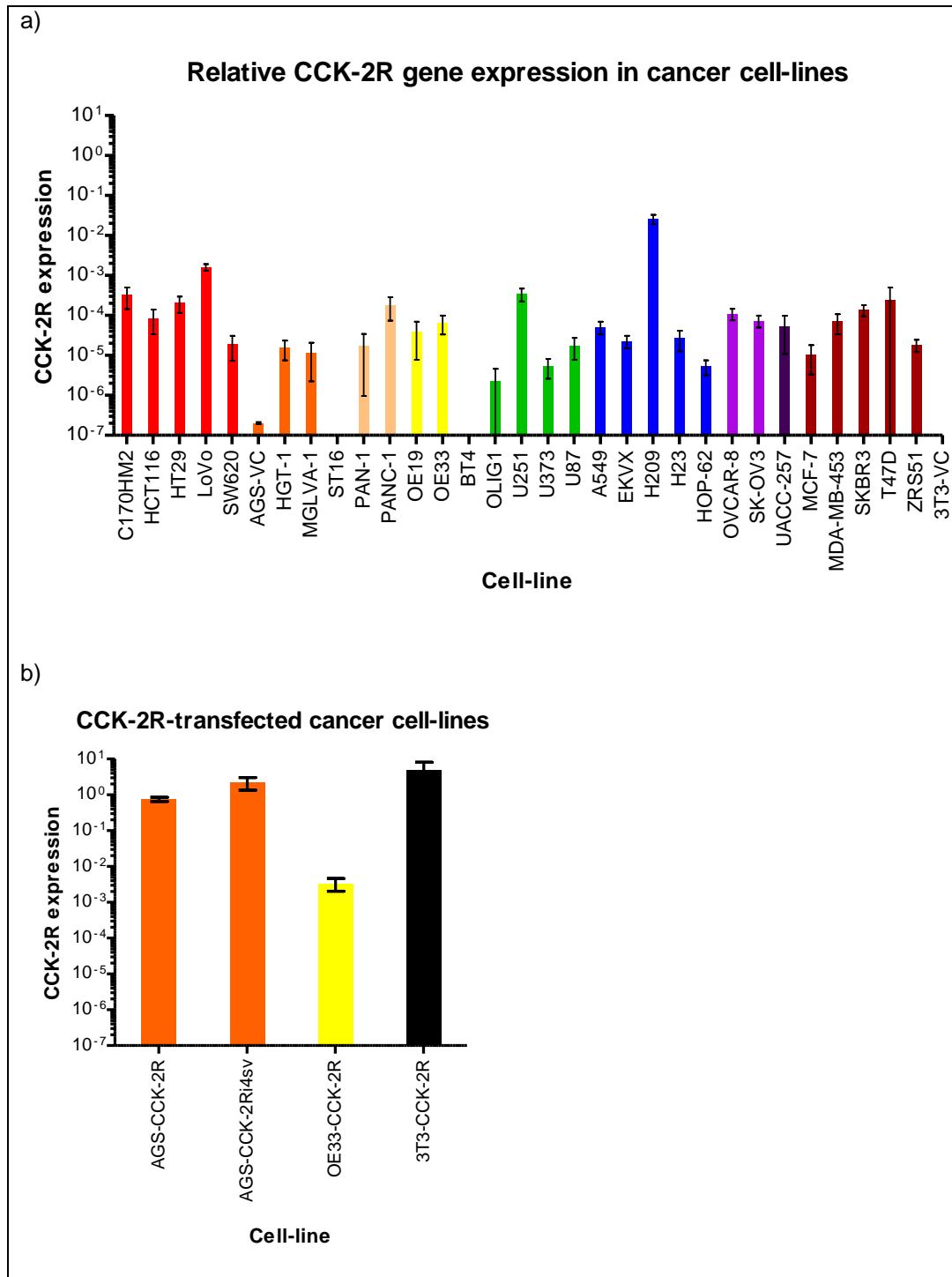
From Figure 3.6 the sensitivity cut-off point for each assay is:

1. Low  $T_m$  LATE-PCR probe at 1:1.0E+06
2. High  $T_m$  LATE-PCR probe at 1:1.0E+04
3. Taqman qRT-PCR at 1:1.0E+02.

This means that the best LATE-PCR assay is 10,000-fold more sensitive than the CCK-2R Taqman qRT-PCR.

### **3.6. CCK-2R gene expression in a cell-line panel using LATE-PCR**

LATE-PCR was used to investigate CCK-2R mRNA expression in the panel of cell-lines tested above (Section 3.2), plus additions, to determine whether this more sensitive method enabled detection of CCK-2R in cell-lines which were negative by the Taqman assay, but in which biological assays had suggested that the receptor was expressed [13, 74, 138, 139].

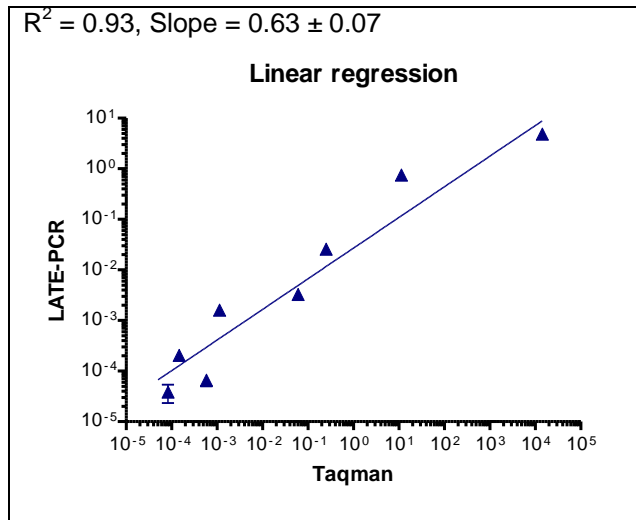


**Figure 3.8: Logarithmic graph showing the relative expression of CCK-2R ( $2^{-\Delta Ct}$ ) in a panel of cell-lines, using LATE-PCR**

A range of cancer types are represented on separate graphs; a) endogenous expression and b) stably-transfected cell-line results. Each cell-line was assayed in triplicate using cDNA from 2 different passages of the cell-line, representing independent replicates and the black bars represent the SEM (one-way ANOVA showed none of the bars in each graph to be significantly different to any other).

Figure 3.8 illustrates the results of the LATE-PCR assay on a cancer cell-line panel. Most of the cell-lines tested did express CCK-2R;

however the level of that expression varies dramatically, shown by the log scale of the graphs. Each cancer group, e.g. colorectal, does not contain enough samples to show a trend, however, there are some individual cell-lines which contain a high endogenous level of CCK-2R, for example H209 (SCLC). This cell-line correlates to the high expression shown in the Taqman assay, above, as do the other cell-lines tested in both assays ( $p < 0.001$ ; Figure 3.9).



**Figure 3.9: Linear correlations performed on the CCK-2R expression data obtained using Taqman qRT-PCR and LATE-PCR**

*The  $R^2$  value and slope are described above the graph, and the error bars represent the SEM of the data sets.*

The colorectal cell-lines in Figure 3.8 show consistently high CCK-2R expression, whereas the gastric cell-lines are the lowest in terms of expression. The lung, pancreatic, oesophageal, melanoma, breast and ovarian cell-lines all show fairly similar levels of CCK-2R expression ( $2^{-\Delta C_t}$  at  $\sim 10^{-4}$ ). The exception to this is H209 cells with 100-fold higher expression, the second highest level in the cell-line panel tested, but this was expected since they have been previously shown to express CCK-2R by our and other groups [159]. Also the glioma cells show considerable variation, from no expression in BT4 cells to  $\sim 10^{-4}$  in U251 cells.



A panel of stably-transfected cell-lines was also tested in LATE-PCR and shown in Figure 3.8b. The transfected AGS cell-lines showed far more gene expression than the vector controls shown in Figure 3.8a ( $1 \times 10^7$ -fold,  $p < 0.0001$ ), and the OE33-CCK-2R cell-line had 100 times more activity than the wild-type OE33 cells in Figure 3.8a ( $p < 0.01$ ). Vector control mouse NIH3T3 cells had no expression of human CCK-2R as anticipated, but the transfected NIH3T3 cell-line showed high expression (Figure 3.8).

Each cell-line was tested four times (two pellets from two passages being the closest to independent replicates possible), and each cell-line shows no significant difference between these replicates by the one-way ANOVA statistical test.

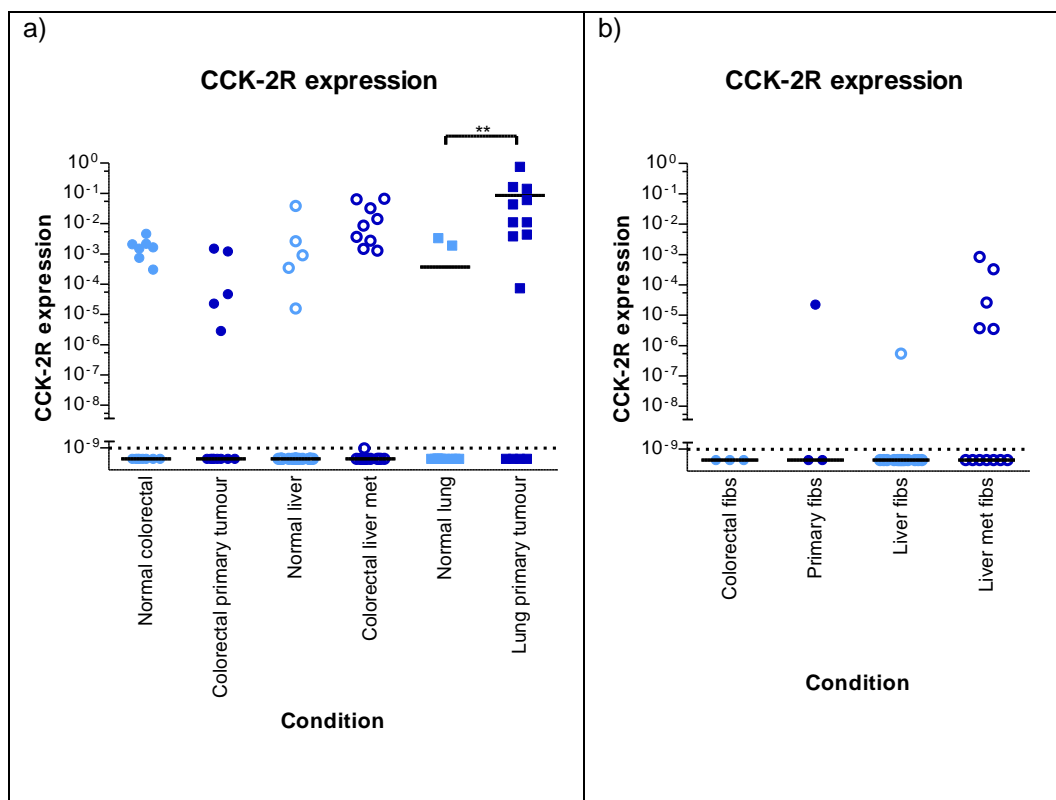
While testing these cell-lines, gDNA contamination sometimes became a problem in LATE-PCR, especially in cell-lines where CCK-2R expression was low. In order to combat this, a special cDNA synthesis kit was used for reverse transcription, the Qiagen QuantiTect Reverse Transcription kit. This kit includes a step to remove gDNA before starting the cDNA synthesis reaction. Also the LATE-PCR conditions were changed slightly, eliminating the extension and detection step from the first ten cycles, in order that any gDNA still present would not be amplified.

### **3.7. CCK-2R expression in primary tumour samples**

CCK-2R gene expression has been demonstrated in many human cancer cell-lines, but in order to confirm the value of LATE-PCR, it was also tested on primary tumour samples. A number of primary tumour and adjacent normal samples were obtained and used under ethical approval from Nottingham Research Ethics Committee, Reference 08/H0403/37. These samples were biopsies taken by surgeons at

Nottingham University Hospitals. The normal tissue; colonic epithelium, liver epithelium or lung parenchyma, was taken from at least 50mm away from the tumour site and any large blood vessels were removed before processing. Solid tumour and normal sample RNA was extracted by Dr Richard Argent and Mr Alexander Reece-Smith, who also performed cDNA synthesis. In the current project these cDNA samples were analysed by LATE-PCR for CCK-2R expression. The purpose of this study was to validate LATE-PCR in primary samples and to correlate gene expression between

- normal and tumour tissue
- primary tumour site and corresponding metastasis
- whole tumour tissue and cancer-associated fibroblast cells.



**Figure 3.10: CCK-2R gene expression in tumour samples**

Logarithmic dot plot showing the expression of CCK-2R (2<sup>-ΔCt</sup>) in a) normal and tumour tissue of the colon primary, liver metastasis from colon and lung primary, and b) with colon and liver fibroblasts (fibs) selected for from the samples and tested separately. The black line represents the median for each subset (\*\* indicates  $p < 0.01$ ).

Samples	Number of samples	Proportion of positive values	Proportion of positive values
Colorectal	15	46.7%	36.0%
Primary tumour (colo)	13	38.5%	
Liver	30	16.7%	
Liver met from colo	25	40.0%	
Lung	14	14.3%	
Lung primary	14	71.4%	
Colorectal fibs	3	0.0%	22.0%
Primary fibs	3	33.3%	
Liver fibs	14	7.1%	
Liver met fibs	12	41.7%	

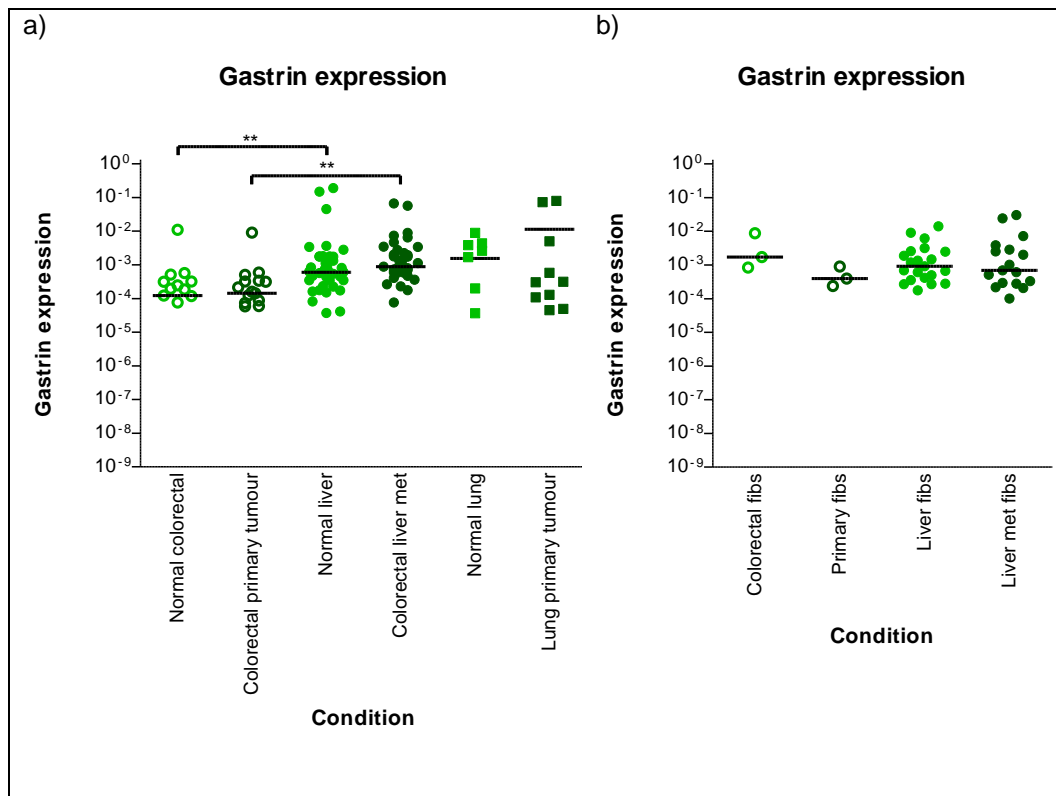
**Table 3.3: Number of sample and the proportion of positive values in each of the sample groups displayed in Figure 3.10**

Figure 3.10 shows the CCK-2R gene expression levels in normal tissue and tumour tissue (mixture of epithelial and fibroblast cells) of the colon, liver and lung (a), and also in the fibroblast complement of the colon and liver (b). The samples below the dotted line in both graphs in Figure 3.10 represent the negative samples, where CCK-2R expression is below the detection of the LATE-PCR assay. This was determined from the sensitivity cut-off points described in section 3.8 above, at around  $1 \times 10^6$ . The total number of samples tested is shown in Table 3.3, giving an indication of the number of negative values shown on each graph in Figure 3.10. The proportion of positive expression values in the whole tumour tissue data is 36% (Table 3.3), which is around average for the individual data sets, except the lung primary tumour where 71.4% of the samples were positive for CCK-2R.

Using the one-way ANOVA the lung primary tumour sample shows significantly higher (100-fold) CCK-2R expression to that of the normal lung. The other pairs were shown to be statistically similar. The fibroblast samples were also shown to be similar to that of whole tissue, although there are not enough samples in these groups to perform a proper calculation. Gastrin gene expression was investigated in the same samples (Figure 3.11), in order to perform a correlation.

### 3.7.1. Gastrin expression

The one-way ANOVA test shows significantly higher gastrin gene expression in the normal and metastatic liver tissue compared to the equivalent colorectal samples in Figure 3.11. The difference between the gastrin expression in colorectal primary and liver metastasis tissues is negated by the fact that the normal liver shows higher expression than the colon. None of the other samples were statistically different from each other. The number of samples in each group in Figure 3.11 is shown in Table 3.3, since they are the same samples as those used for Figure 3.10.



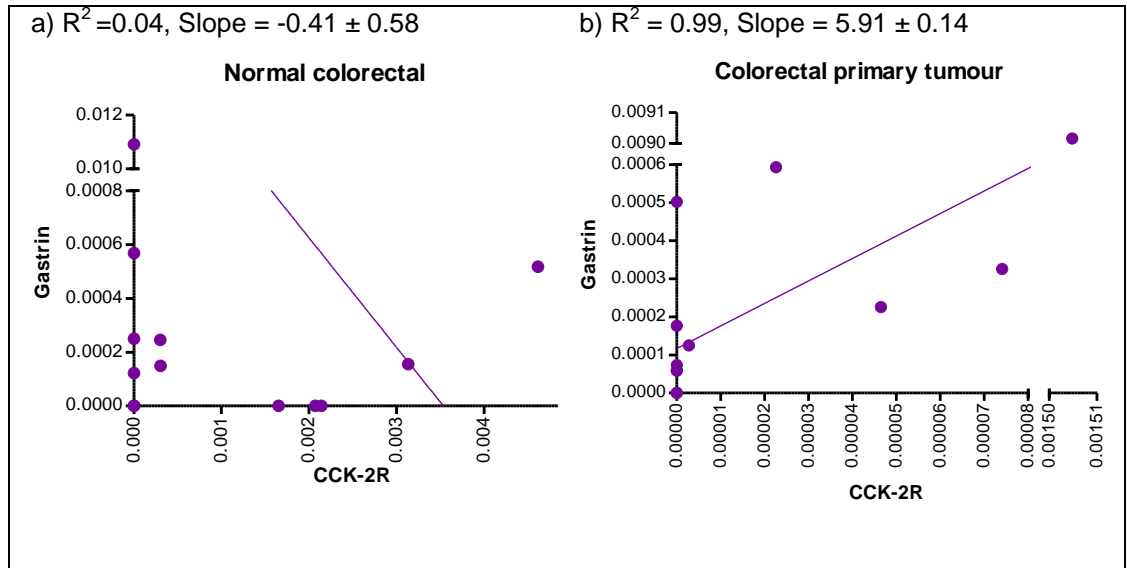
**Figure 3.11: Gastrin gene expression in tumour samples**

Logarithmic dot plot showing the expression of gastrin ( $2^{-\Delta C_t}$ ) in a) normal and tumour tissue of the colon primary, liver metastasis from colon and lung primary, and b) with colon and liver fibroblasts (fibs) selected for from the samples and tested separately. The black line represents the median for each subset (\*\*indicates  $p < 0.005$ ).

### 3.7.2. The correlation between CCK-2R and gastrin expression

Linear regression analysis carried out in GraphPad Prism showed a positive correlation between CCK-2R and gastrin expression in the

primary colorectal tumour by linear regression analysis ( $p < 0.0001$ ; Figure 3.12). The normal colorectal scatter graph is shown as an example of non-correlated data ( $p = 0.50$ ), and the liver and lung samples were not shown since these also lacked correlation.



**Figure 3.12: Correlation between CCK-2R and gastrin expression in tumour samples**

Scatter plots showing the linear regression lines between CCK-2R and gastrin gene expression in the tumour samples; a) normal colorectal, b) primary colorectal tumour. The  $R^2$  value and slope are described above the graph, showing that the correlation is significant in b) ( $p < 0.0001$ ).

The normal colorectal samples showed no correlation, which is interesting in itself, since tumourigenesis presumably causes the relationship between CCK-2R and gastrin gene expression to develop. Grade of tumour (data available for colorectal and liver), age/gender of the patient (all samples) and smoker status (lung alone) data was converted to a number rank in order to correlate it with CCK-2R gene expression. An example of this conversion can be demonstrated using grade of tumour where grade A = 1; grade B = 2; grade C1 = 3 etc. These calculations, however, produced no correlations. None of the patients had been treated with chemotherapy for their cancer, so a correlation with drug use could not be investigated.

### **3.8. Validation of LATE-PCR using siRNA molecules directed towards the CCK-2R**

In order to validate LATE-PCR as a method of detecting the CCK-2R, the receptor was knocked-down in several cell-lines using siRNA. If LATE-PCR could show that the CCK-2R had been down-regulated in these cell-lines then it would not only demonstrate its specificity for CCK-2R but also that LATE-PCR is sensitive enough to detect a change in the, already low, CCK-2R expression shown in some cells.

#### **3.8.1. siRNA design**

RNA interference was employed in order to study the effects of knocking down the CCK-2R. siRNAs were designed to target different regions of the receptor, using a number of algorithms available on the internet (Dharmacon [165], Genscript [166] and siSEARCH). Each yielded a list of potential siRNAs, in order of worth according to the conditions stipulated by the algorithm; for example if a siRNA needed sense strand modification to reduce sense strand uptake this would be ranked lower by the Dharmacon algorithm (Table 3.4). For the CCK-2R, a list of possible siRNAs was obtained using each of these algorithms (Table 3.4), and then compared to each other. Only Dharmacon and Genscript produced consistent sequences so these were used and the siSEARCH siRNAs do not appear in Table 3.4, which lists the siRNA sequences and features.

The human CCK-2R gene is 2,120bp long and the extreme 5' and 3' ends of the sequence were avoided due to lack of conserved residues and the risk of polymorphisms being present [165]. Four siRNAs were chosen, to target sequences around 300bp apart along the gene (Table 3.4; CCK1, 2, 3 & 4).

siRNA sequence	Dharmacon (score)	Low seed freq?	Need sense mod?	Genscript (score)	Start position	G/C content	Name of siRNA
<b>GAGAATTGGA GCTGGCCAT</b>	Yes (2 <sup>nd</sup> )	No	No	Yes (1 <sup>st</sup> )	342	53%	<b>CCK1</b>
<b>GATGAGCGTT GGAGGAAAT</b>	Yes (1 <sup>st</sup> )	No	No	Yes (2 <sup>nd</sup> )	391	47%	
<b>GCTTCGCTGT CCAGGCTTA</b>	Yes (22 <sup>nd</sup> )	No	No	Yes (3 <sup>rd</sup> )	1484	58%	
<b>AGTGACAGCG ACAGCCAAA</b>	Yes (16 <sup>th</sup> )	No	No	Yes (6 <sup>th</sup> )	956	53%	<b>CCK2</b>
<b>TGCGAATGTT GCTGGTGAT</b>	Yes (50 <sup>th</sup> )	No	No	Yes (7 <sup>th</sup> )	1188	47%	
<b>TCATTCAGTTG CTGAGCTA</b>	Yes (17 <sup>th</sup> )	No	No	Yes (8 <sup>th</sup> )	1314	42%	<b>CCK3</b>
<b>CCATCGCACT GGAGCGGTA</b>	Yes (3 <sup>rd</sup> )	Yes	No	No	633	63%	<b>CCK4</b>

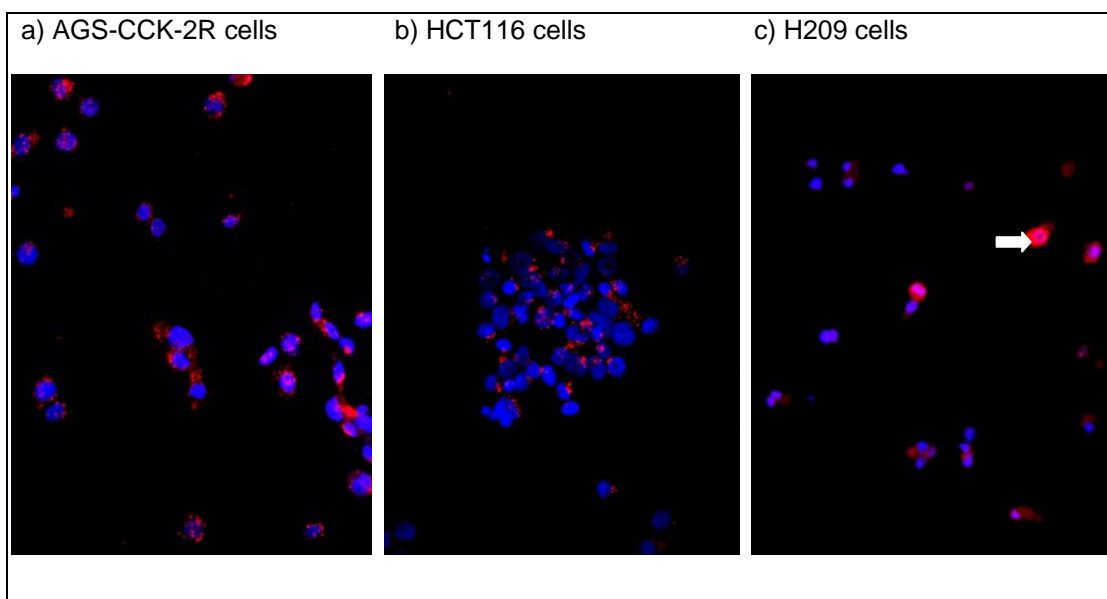
**Table 3.4: Table showing the comparison of siRNA sequences from the algorithms of Dharmacon and Genscript.**

*The last column indicates those siRNAs ordered and tested by transfection into AGS-CCK-2R cells.*

### 3.8.2. siRNA transfection efficiency

In order to investigate knock-down of the receptor, these siRNAs were transfected into those cells showing expression of the CCK-2R in the LATE-PCR assay. Before transfecting the cells with the CCK-2R siRNAs, the transfection efficiency was examined using a fluorescent TAMRA-labelled siRNA. The cells were visualised under a fluorescent microscope, with a blue nuclear stain, Hoechst. Where the siRNA had entered the cells, it was revealed as red fluorescence. By measuring the percentage of nuclei with associated siRNA, the transfection efficiency was determined in AGS-CCK-2R, HCT116 and H209 cells (Figure 3.13) and in the other cells to be transfected (not shown). The AGS-CCK-2R and HCT116 cells were transfected using the standard siPORT Amine protocol, whereas the H209 cells were reverse transfected using the same transfection agent because these cells grow in suspension; however, it seems to be as effective as the standard protocol for the other cell-lines. The distribution of the siRNA between cells is similar in HCT116 and AGS-CCK-2R populations, whereas in

H209 cells it appears that some cells have more siRNA within them: the red fluorescence is brighter around their nuclei (white arrow in Figure 3.13).



**Figure 3.13: TAMRA-siRNA transfected cells**

*a) AGS-CCK-2R cells, b) HCT116 cells, c) H209 cells, all transfected with a fluorescent TAMRA-labelled siRNA. The siRNA is red, and the nuclei have been stained blue with Hoechst. The first two cell-lines were transfected using the standard protocol, whereas the H209 cells were reverse transfected as they grow in suspension. The white arrow indicates a H209 cell with more siRNA within it than the average.*

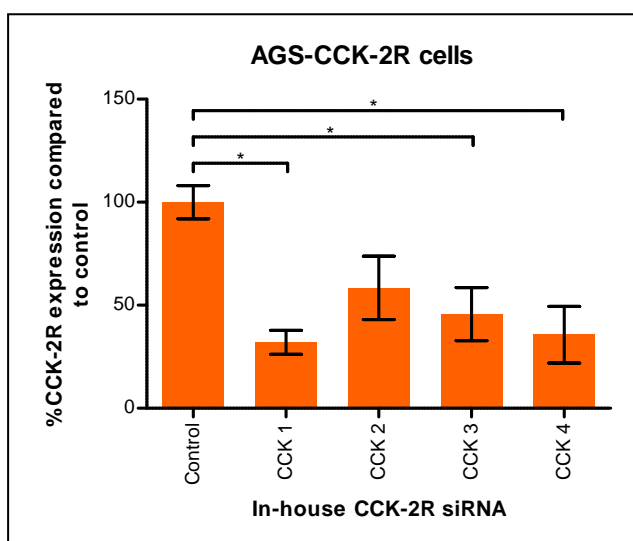
Figure 3.13 illustrates that the transfection efficiency was around 90% in the three cell-lines shown above and this was the case in the other cell-lines tested, except for SW620 cells where it was around 50%. It was assumed that the CCK-2R targeted siRNAs would enter the same percentage of cells as the TAMRA-labelled siRNA.

### **3.9. Knockdown of the CCK-2R in AGS-CCK-2R cells**

The aim of developing CCK-2R-targeted siRNAs was to reduce receptor expression, to validate the LATE-PCR assay and understand what effects knock-down has on the cell. Four CCK-2R siRNAs were made in-house by annealing sense and antisense RNA oligonucleotides. These were transfected into AGS-CCK-2R cells, along



with a negative siRNA, and analysed by Taqman qRT-PCR, since receptor expression in these cells should be high enough to be detected by this assay. LATE-PCR was used if the Taqman assay became inadequate. The negative siRNA should not have targeted any genes, and so should control for any change which is due to the transfection reagent, siPORT Amine, or non-specific effects of siRNA. Three of the CCK-2R siRNAs produced significant knock-down, compared to the negative control. CCK1 was the most successful siRNA, reducing expression of CCK-2R to 32% ( $p < 0.05$ ) of that in the scrambled siRNA-transfected control, whereas the others only resulted in reduction to 40 and 65% of the control expression (Figure 3.14).



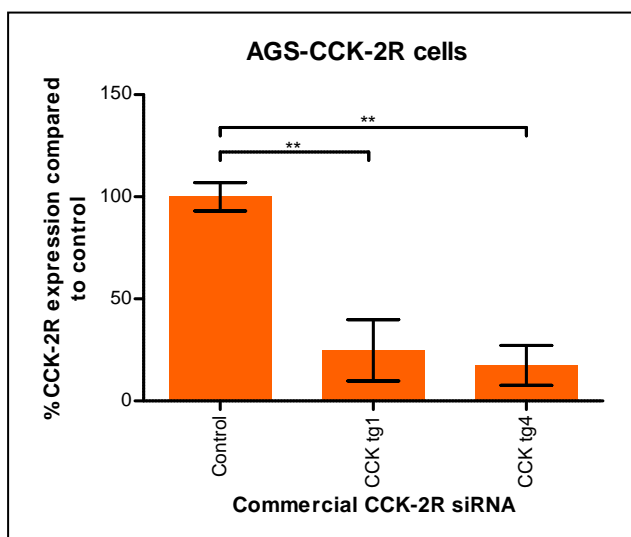
**Figure 3.14:** Percentage expression of the CCK-2R in AGS-CCK-2R cells when transfected with in-house siRNAs designed to target the receptor

*The method of detection of the CCK-2R is Taqman qRT-PCR, and the results are an average of three repeats. The siRNAs CCK1 and 4 are most effective at down-regulation; all three CCK-2R siRNAs cause significant knockdown (\* indicates  $p < 0.05$ ) compared with the negative control siRNA. The black bars represent the standard error of the mean (SEM).*

CCK4 was also able to produce satisfactory knock-down to 36% of the control (Figure 3.14;  $p < 0.05$ ). One of the final two CCK-2R siRNAs did produce significant down-regulation (CCK3: 46% of control;  $p < 0.05$ ) but was not investigated further, due to having less activity than CCK1 and 4.

Since siRNAs made in-house from oligonucleotides are usually found to be less effective than those purchased commercially, the two most efficient siRNAs were ordered for further studies. CCK1 and CCK4 were commercially synthesised as CCK tg1 and CCK tg4, respectively, and

these were once more transfected into AGS-CCK-2R cells. Figure 3.15 shows that both commercially-made siRNAs are more effective at down-regulating the CCK-2R compared to the corresponding in-house siRNA.



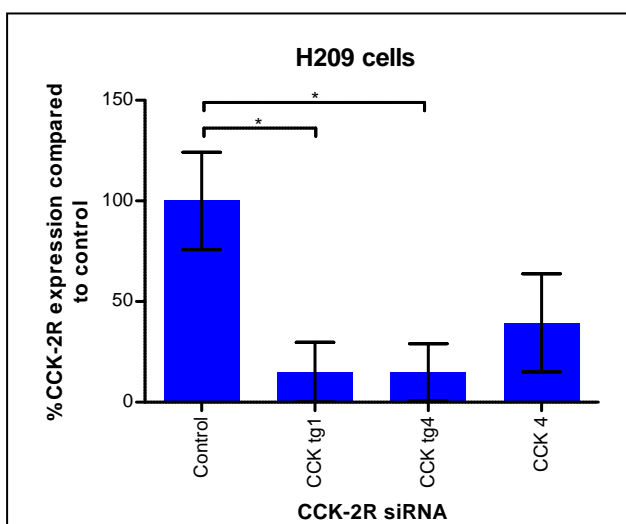
**Figure 3.15: Percentage expression of the CCK-2R in AGS-CCK-2R cells when transfected with commercial siRNAs targeting the receptor**

*The method of detection of the CCK-2R is Taqman qRT-PCR, and the results are an average of three repeats with the black bars representing the SEM. Both siRNAs cause significant knockdown (\*\* indicates  $p < 0.005$ ).*

The commercially-made siRNAs reduced CCK-2R expression to 17% (CCK tg4;  $p < 0.005$ ) and 25% (CCK tg1;  $p < 0.005$ ) of control siRNA-transfected cells. This is an improvement on the result in Figure 3.14, where the equivalent in-house siRNAs caused 36% and 32% expression respectively. Since these siRNAs are obviously causing down-regulation of the CCK-2R mRNA in a stably transfected cell-line, they were then tested in cells showing endogenous receptor expression.

### 3.9.1. Knock-down of the CCK-2R in other cells

The two commercial CCK-2R siRNAs were initially transfected into the SCLC cell-line, H209, since these cells have been shown to express the highest level of endogenous CCK-2R in the cell-line panel screened. H209 cells also demonstrated excellent transfection efficiency (Figure 3.13) in this experiment, despite the reverse transfection method necessary for these suspension cells, and so the samples were tested by LATE-PCR for knock-down of the CCK-2R (Figure 3.16).



**Figure 3.16: Percentage expression of the CCK-2R in H209 cells when transfected with commercial (CCK tg1 & 4) and in-house (CCK4) siRNAs**

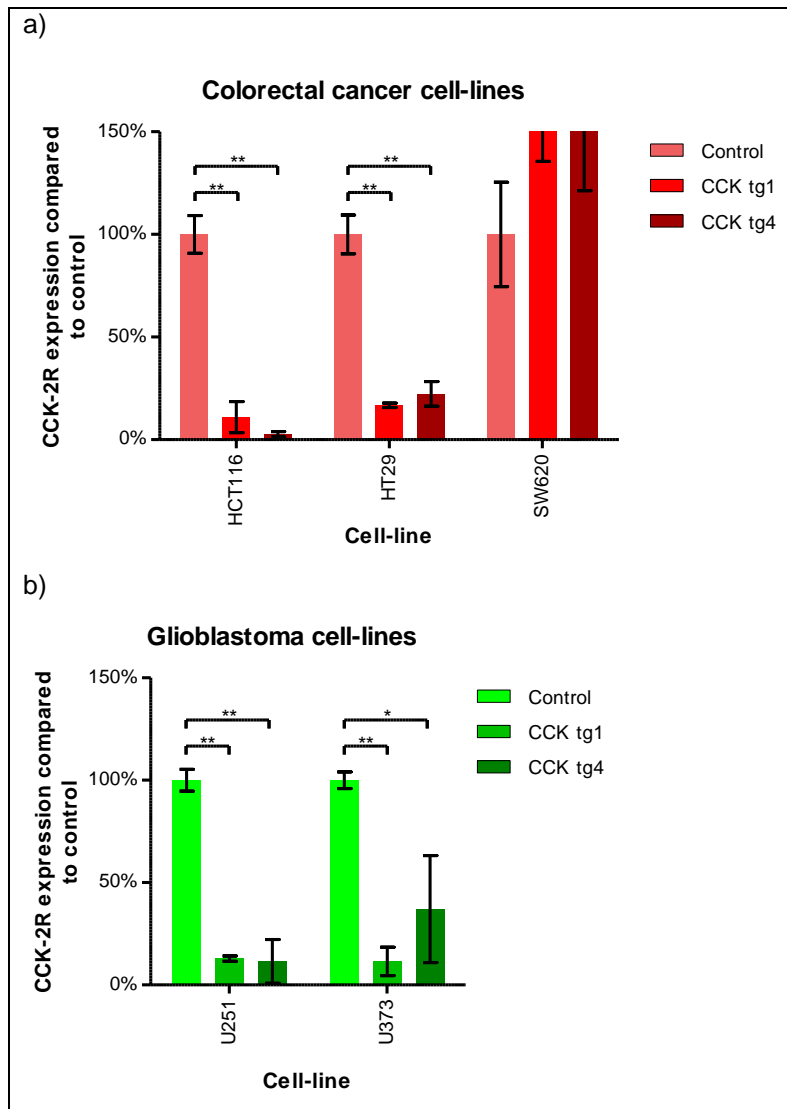
*The method of detection of the CCK-2R is LATE-PCR, and the results are an average of three repeats with the black bars representing the SEM. The commercial siRNAs are most effective at down-regulation (\* indicates  $p < 0.05$ ).*

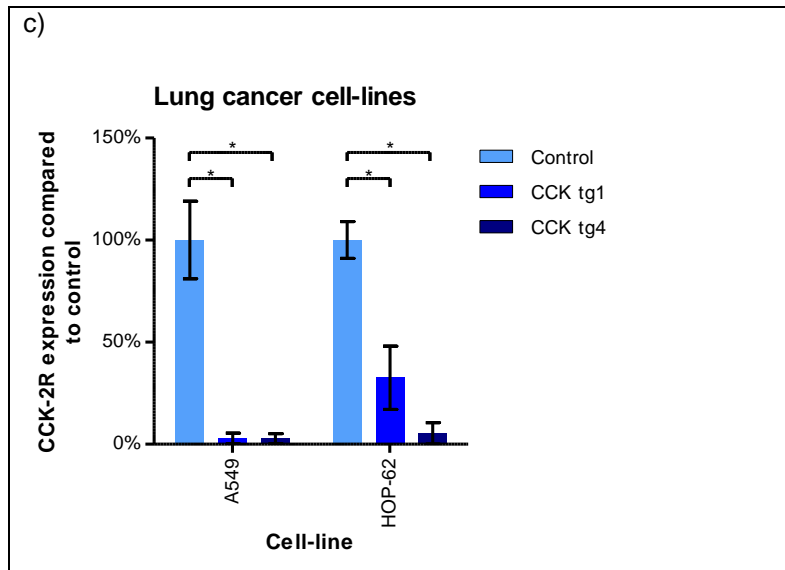
These results confirm that the CCK-2R siRNAs result in knock-down of the receptor in H209 cells, with both commercial siRNAs reducing expression to 15% of control expression ( $p < 0.05$ ). This is similar to that seen in AGS-CCK-2R cells (Figure 3.15) above. Figure 3.16 shows that no significant knockdown was achieved when the CCK4 siRNA made in-house was used.

The same cDNA samples were tested using the Taqman assay, but the results were inadequate. The fact that LATE-PCR could detect siRNA activity, with similar trends to those shown in AGS-CCK-2R cells, i.e. the commercial siRNAs produce more down-regulation than the in-house versions, is further proof that this new assay is far more sensitive.

Since the LATE-PCR could detect CCK-2R expression in siRNA-transfected cells with high endogenous expression, this method was tested in a panel of cell-lines. This panel was chosen as a subset of that above, using a high, medium and low-expressing cell-line within the colorectal, glioma and lung cancer cell-lines. Colorectal cell-lines showed mixed results (Figure 3.17a), with good knock-down by both commercial siRNAs in HCT116 and HT29, but no down-regulation in

SW620 cells. Figure 3.8a indicates that the CCK-2R expression levels in each of these cell-lines are similar. Transfection efficiency was found to be reduced (~50%) in SW620 cells; however this does not account for the total lack of knock-down seen in these cells. HCT116 and HT29 CCK-2R expression could be knocked down to at least 11% and 22% of the control, respectively ( $p < 0.005$ ).





**Figure 3.17: Percentage expression of the CCK-2R in cancer cell-lines when transfected with commercial siRNAs made to target the receptor**

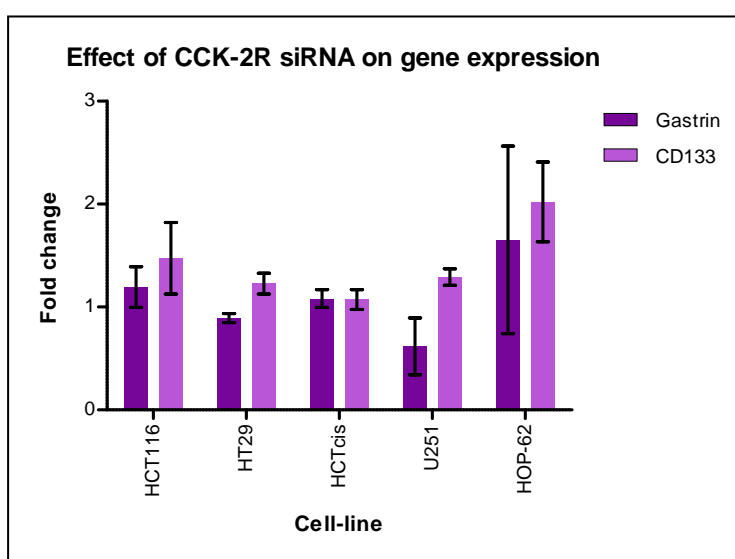
*The graphs show a) colorectal, b) glioma and c) lung cell-lines. The method of detection of the CCK-2R is LATE-PCR, and the results are an average of three repeats with the black bars representing the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.005$ ).*

Two CCK-2R-expressing glioblastoma cell-lines, U251 and U373, were also selected (Figure 3.17b). U373 cells showed good knock-down with the CCK-2R siRNAs especially CCK tg1, which caused expression to drop to 11% of the control level ( $p < 0.005$ ). In U251 cells CCK tg4 allowed only 11% of normal CCK-2R expression, and CCK tg1 knocked-down expression to 13% ( $p < 0.005$ ).

The third set of cells in the panel was the lung cell-lines, of which H209 is shown in Figure 3.16. Both CCK-2R siRNAs cause significant down-regulation of the receptor in A549 and HOP-62 cells; which show 3% and 5% (CCK tg 4) and 3% and 32% (CCK tg 1) expression respectively, all with  $p < 0.05$  (Figure 3.17c). As well as being a method of validation for LATE-PCR, these siRNA molecules were designed to investigate the functional effect of knocking down the CCK-2R.

### 3.10. The effect of CCK-2R knock-down on expression of other genes

Gastrin and CD133 gene expression were investigated in five of the cell-lines shown in Section 3.17 above, which had been transiently transfected with CCK-2R siRNA (CCK tg1) for 24 hours. Gastrin and the CCK-2R are linked in an autocrine loop [137], suggesting that if one is knocked down, the other will be affected, however, this does not seem to be the case here. CD133 expression may have been influenced by CCK-2R knock-down if it was affecting cancer stem cell differentiation.



**Figure 3.18: The effect of knocking down CCK-2R on gastrin and CD133 gene expression**

*The results are the mean of two experiments, and the error bars show the SEM.*

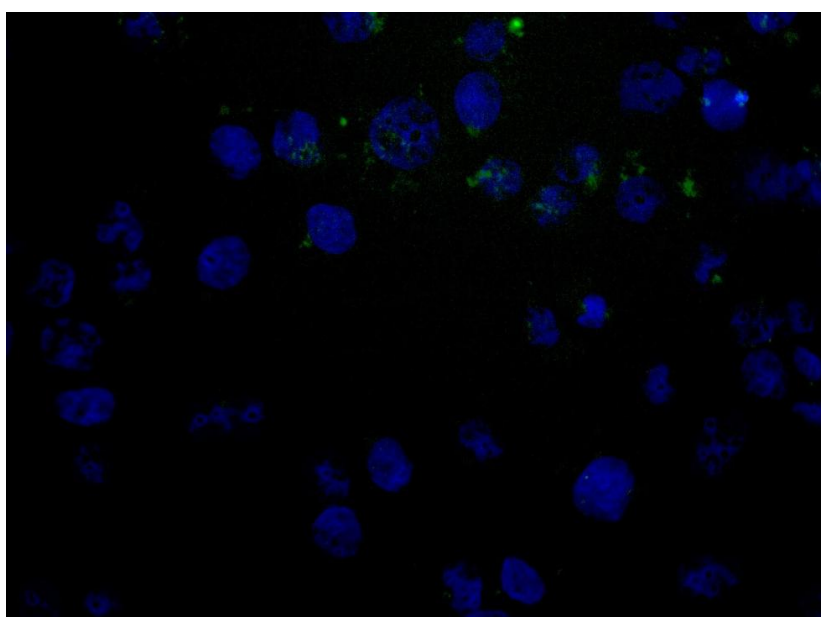
CCK-2R knock-down does not seem to affect gastrin or CD133 gene expression significantly (Figure 3.18).

### 3.11. CCK-2R expression at the single cell level

CCK-2R is up-regulated in cancer cells, and in order to see where it is expressed in these cells an experiment was designed to show the expression in real time. The expression of CCK-2R mRNA could be evenly distributed throughout the cell population, or it could be restricted to certain cells. This question might be answered by transfecting the fluorescent molecular probes designed for LATE-PCR directly into cells,

and visualising them with microscopy and apparatus such as the Flexstation, since they would fluoresce when bound to mRNA.

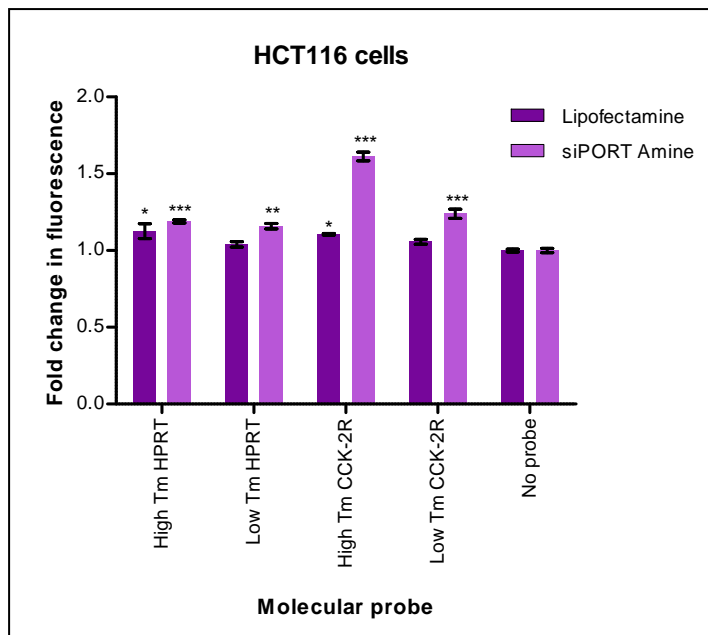
Initially the HPRT LATE-PCR probe was used, since expression of HPRT would be more widespread than that of CCK-2R. 400nmol of probe was added to each well for all the probe transfections described. The first results are shown in Figure 3.19, where the HPRT probe can be seen inside a few of the cells (~20%).



**Figure 3.19: Fluorescent photograph of HCT116 cells transfected with a HPRT LATE-PCR molecular probe**

*The transfection reagent is siPORT Amine, the nuclei are stained with DAPI (blue) and the probe shows as green fluorescence due to its fluorophore.*

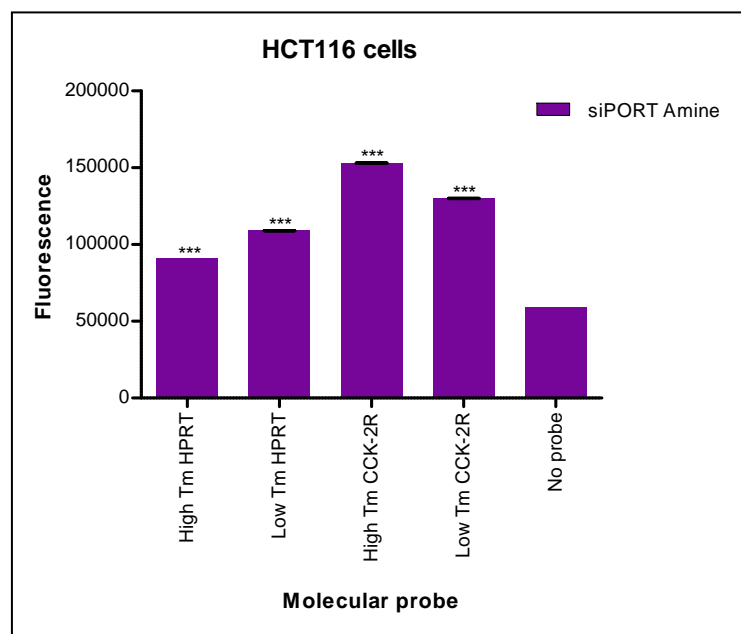
In the second experiment all four LATE-PCR probes (low and high  $T_m$ , HPRT and CCK-2R) were transfected into HCT116 cells, and all four showed significantly more fluorescence than the control with no probe when siPORT Amine was used as the transfection reagent (Figure 3.20).



**Figure 3.20: HCT116 cells transfected with four molecular probes**

The transfection agents used were Lipofectamine and siPORT Amine, and each of the four LATE-PCR probes were transfected separately into HCT116 cells. The data was generated by the Flexstation and normalised to the no probe control sample (value of 1), the error bars represent the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.005$  compared to equivalent no probe control).

Since the siPORT Amine transfection showed the most promise, fluorescence was also assessed using the more sensitive Spectrum machine (Figure 3.21). This confirmed that the best result was gained using the high Tm CCK-2R LATE-PCR probe (not the low Tm probe, which was the best one for LATE-PCR).



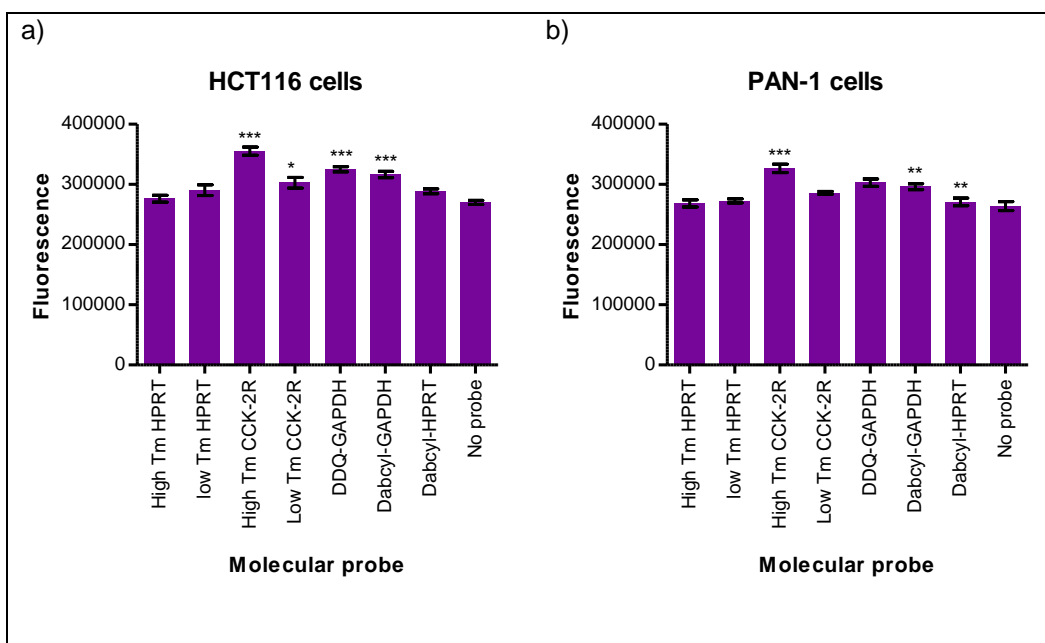
**Figure 3.21: Spectrum data for HCT116 cells transfected with molecular probes**

The transfection agent used was siPORT Amine, and each of the four LATE-PCR probes were transfected separately into HCT116 cells. The data was generated by the Spectrum, the error bars represent the SEM (\*\*\*) indicates  $p < 0.005$  compared to the no probe control).

The fact that CCK-2R was being detected more readily than HPRT was unexpected, since HPRT mRNA was identified sooner than CCK-2R in LATE-PCR. Therefore new molecular probes were designed, with



another housekeeping gene target, GAPDH, and also a new fluorophore, Dabcyl. The DDQ-GAPDH probe shown in Figure 3.22 has the 'deep dark quencher' (DDQ) which is attached to all of the original LATE-PCR probes.



**Figure 3.22: The third molecular probe transfection**

The transfection agent used was siPORT Amine, and each of the seven molecular probes was transfected separately into a) HCT116 and b) PAN-1 cells. The data was generated by the Spectrum, the error bars represent the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.005$  compared to the no probe control).

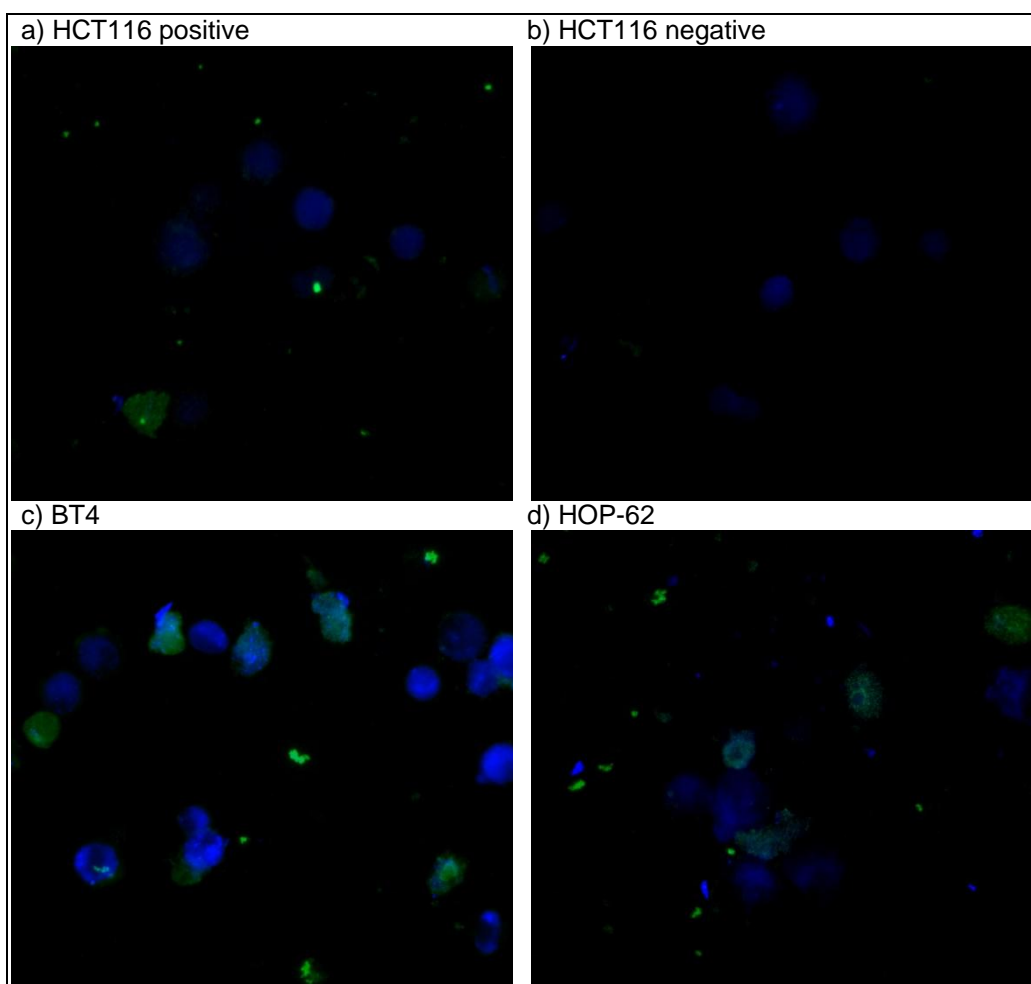
Although the Dabcyl-GAPDH probe was successful in both cell-lines, it still did not surpass the CCK-2R probe in either (Figure 3.22). This method obviously needed further optimisation, possibly by modifying the probes or the transfection reagent, in order to deliver the probes inside the cell more efficiently.

### 3.12. CCK-2R protein expression

Endogenous protein levels of the CCK-2R were investigated in the colorectal, glioma and lung cell-lines, since this is when the CCK-2R facilitates cancer progression and these could be correlated with gene expression. In previous studies, CCK-2R protein was shown indirectly,

by use of inhibitors of gastrin stimulation [13, 74, 138, 139] but in this project it was decided to use a direct method of quantification.

Live cell staining and flow cytometry were used to assess CCK-2R protein expression in the panel of cells. The CCK-2R antibody (Abcam ab14440) chosen for this work binds to the second external loop of the protein (residues 206-219 on Figure 1.7), allowing for live staining. A secondary antibody bound to Fluorescein (FITC) was used to visualise the staining in cytopins (green in Figure 3.23) and quantify expression in the samples by flow cytometry.



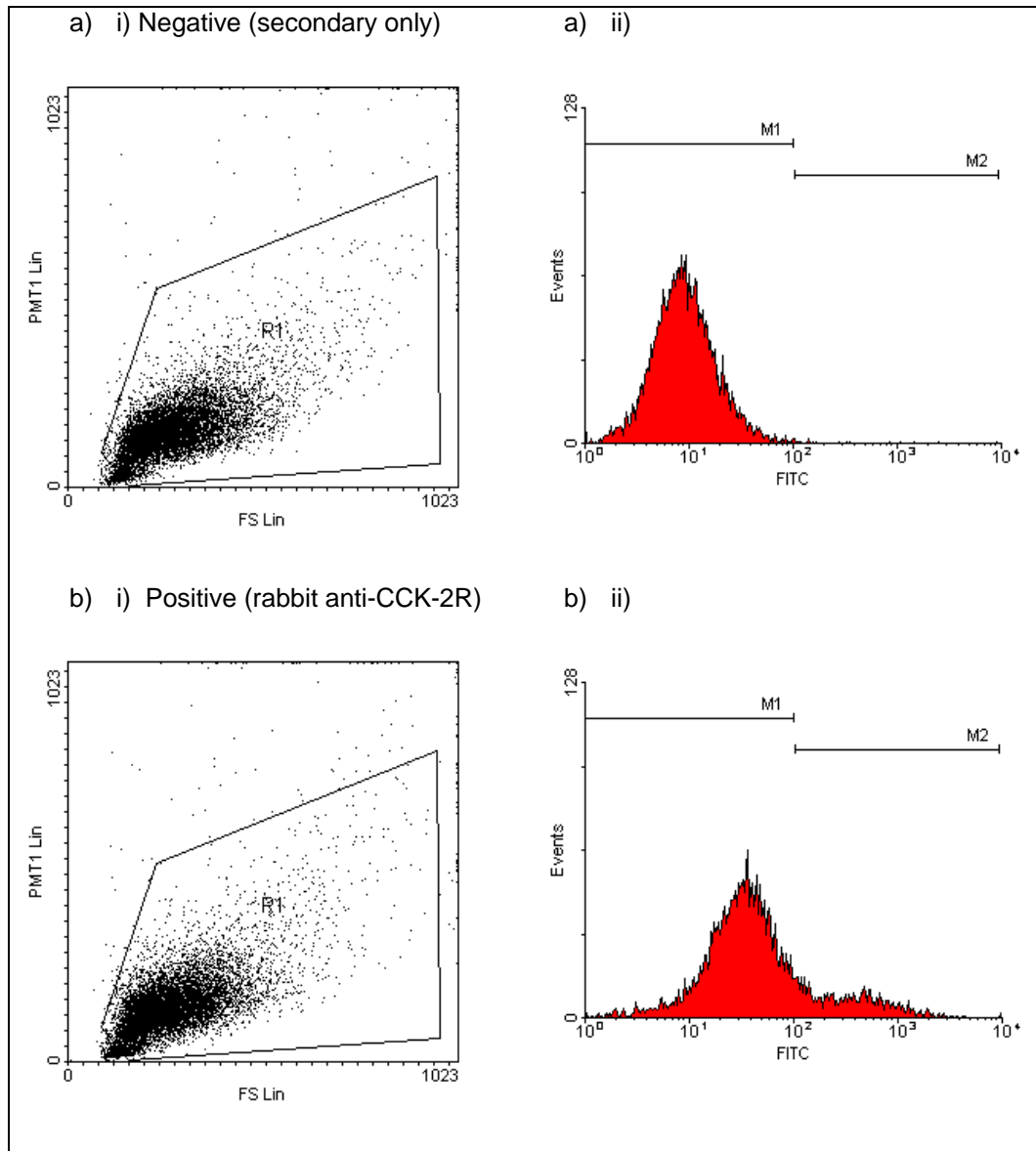
**Figure 3.23: Cytopsin pictures showing CCK-2R live-stained cells**

*The cells were stained with a primary antibody to the second external loop of CCK-2R, and then a secondary antibody attached to FITC (green) unless stated. The cell nuclei were counter-stained with DAPI (blue); a) HCT116, b) HCT116 secondary only, c) BT4 and d) HOP-62.*

Both mouse and rabbit species-specific universal control antibodies were used initially to ensure that the primary antibodies were specific, however, the rabbit universal antibody was depleted during this time and a replacement could not be sourced, so the control for CCK-2R in these experiments was simply secondary antibody alone. The optimal controls for these experiments would be isotype-matched controls, since these would ensure that the control and primary antibodies bound to the same isotype as the primary antibody i.e. IgG. However, it is not necessary to use these in this experiment since, for example, the mouse universal control antibody binds to IgG1, the isotype of the CD133 antibody used.

From the cytospin pictures in Figure 3.23, it would appear that the percentage of highly-stained cells is around 30% for HCT116, 35% for BT4 and 50% for HOP-62 cells. The negative control in Figure 3.23b shows no FITC expression at all, showing that the secondary antibody has bound only when the primary antibody was added; to the positive samples.

Flow cytometry data was analysed in WinMDI, where a dot plot and histogram were drawn (Figure 3.24) and the statistics determined (Figure 3.25a & b). The dot plots shown in Figure 3.24ai & bi demonstrate forward light scatter (FS lin) against side light scatter (PMT1 lin), in order to get a representation of the physical properties of the cells.



**Figure 3.24: Representing the data gained from flow cytometry**

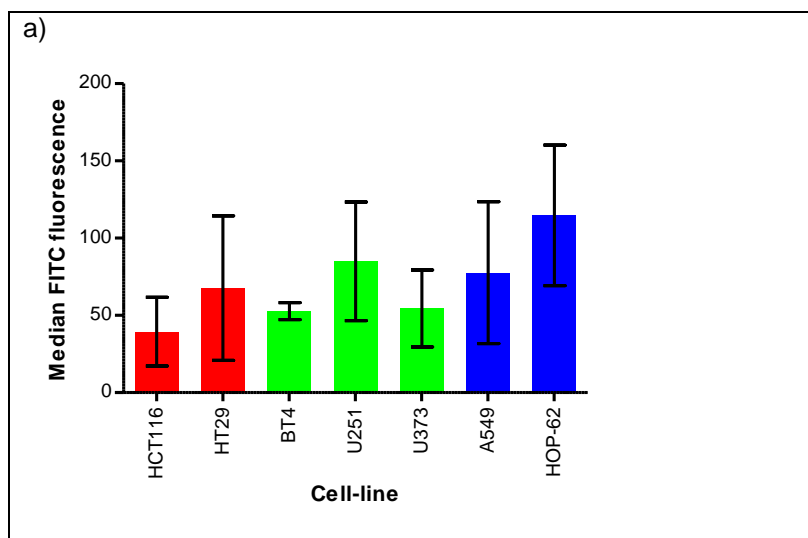
The antibodies used were a) secondary only and b) rabbit anti-CCK-2R followed by secondary shown by i) dot plot of PMT1 Lin against FS Lin or ii) histogram of FITC expression. R1 indicates the gating used to remove dead cells from the histogram data. The statistics taken from the histogram; median of the whole population to show the shift between negative and positive, and the proportion of cells under marker 2 (M2), which shows the highly positive cells.

The R1 region was created to remove dead cells from the analysed population and this was used to gate the histograms in Figure 3.24aii & bii. The histograms were drawn to show the number of cells (events) displaying FITC fluorescence at increasing intensity across the x-axis. From the histogram, two statistics were obtained and used to generate the graphs in Figure 3.25; the median fluorescence of the whole

population, and the percentage of highly-positive cells; under marker 2 (M2).

In Figure 3.24aii & bii it was observed that the peak of the histogram (M1) shifts right from  $10^1$  to half-way between  $10^1$  and  $10^2$ . This indicates that the majority of cells express the CCK-2R (CCK-2R<sup>lo</sup>). In addition, there is a discrete second population of cells (CCK-2R<sup>hi</sup>), with fluorescence 10-fold higher than that of the majority of cells (Figure 3.24bii).

The data was normalised using the negative control (secondary antibody only) values. The median FITC fluorescence (Figure 3.25a) shows the relative levels of protein expressed by the whole population of cells, which varied extensively between experiments (large error bars). The percentage of highly-positive cells shown in Figure 3.25b, however, was more reproducible and demonstrates that glioma and lung cells have a higher proportion of CCK-2R<sup>hi</sup> cells than colorectal cells.



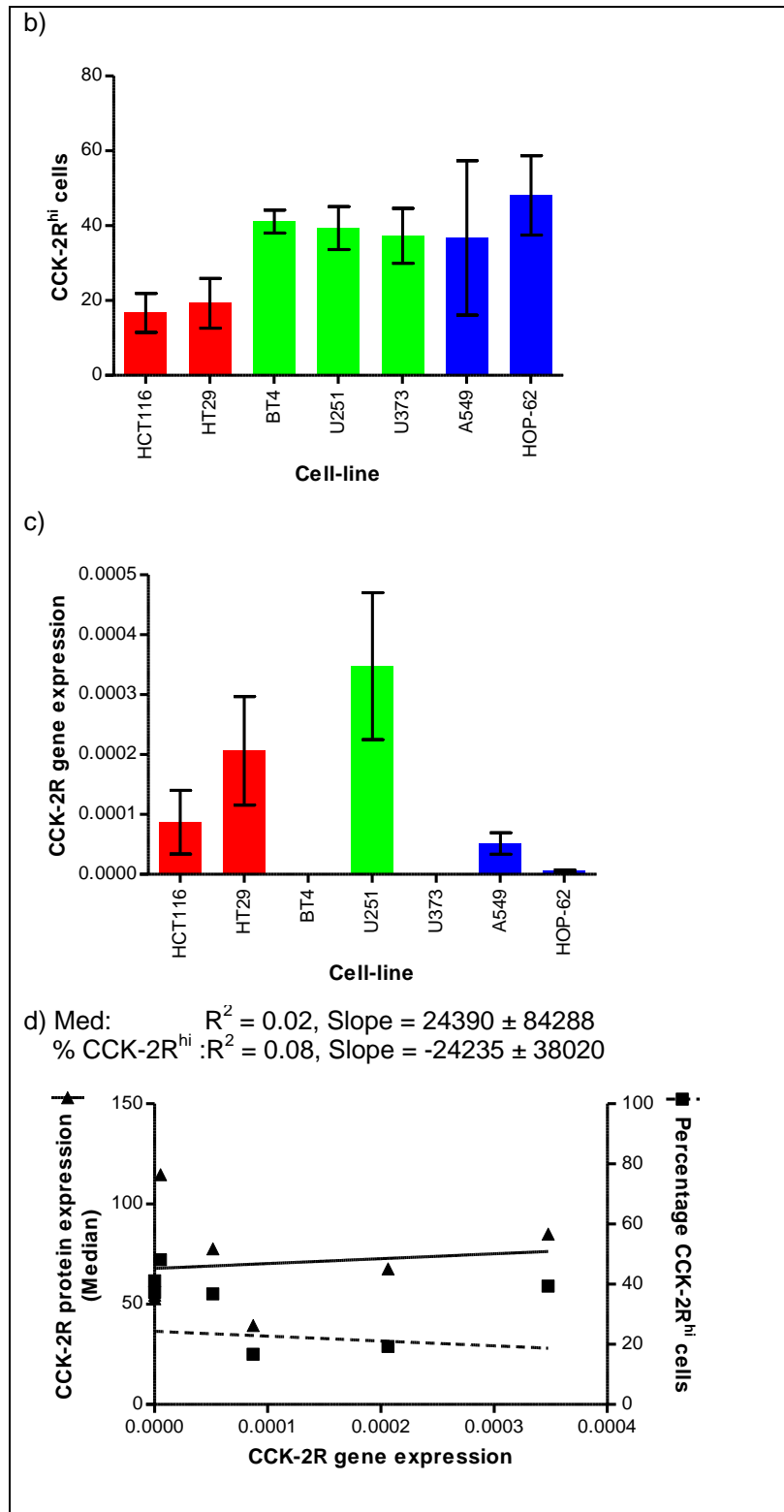


Figure 3.25: CCK-2R protein and gene expression in seven cell-lines

Colorectal cell-lines are shown in red, glioma in green and lung in blue. Protein expression was measured using flow cytometry; a) median FITC fluorescence and b) percentage highly-positive cells (the black bars represent the SEM of three experiments). Gene expression is shown again (from Figure 3.8) in c) but on a linear scale, and d) illustrates the linear regression line between both measures of protein expression and gene (not significant;  $p > 0.5$ ).

Figure 3.25c illustrates the gene expression data shown in a logarithmic graph in Figure 3.8, on a linear scale, in order to compare it to the protein expression data. The linear regression lines between both protein measurements and the gene levels are not significant (Figure 3.25d).

### 3.13. Summary

- The LATE-PCR assay is 10,000-fold more sensitive than the CCK-2R Taqman qRT-PCR.
- Use of LATE-PCR demonstrates that CCK-2R mRNA is present in many cancer cell-lines, where previously only protein expression had been detected.
- LATE-PCR has also shown CCK-2R gene expression to be present in cancer cells where it has not been found before, such as NSCLC and glioblastoma cell-lines.
- 36% of the primary tumour, and their equivalent normal, tissues demonstrated CCK-2R gene expression.
- Lung tumour samples showed significantly higher expression of CCK-2R than the lung normal tissue.
- There was a correlation between CCK-2R and gastrin gene expression in primary colorectal tumour, normal liver and liver metastatic tumour tissue.
- CCK-2R siRNA molecules have been used to verify the specificity of LATE-PCR.
- CCK-2R knock-down in cells did not have an effect on gastrin or CD133 gene expression.
- Molecular probe transfection into cancer cell-lines needs to be optimised but showed initial promise, when the probe could be seen inside HCT116 cells.
- A discrete population of highly CCK-2R-positive cells was present in each cell-line.

- CCK-2R gene expression could not be correlated with protein expression, whether this was defined as the average CCK-2R protein expression per cell or the percentage of CCK-2R highly-positive cells in the population, suggesting there may be translational regulation of this gene.



## Chapter 4 The regulation of CCK-2R expression

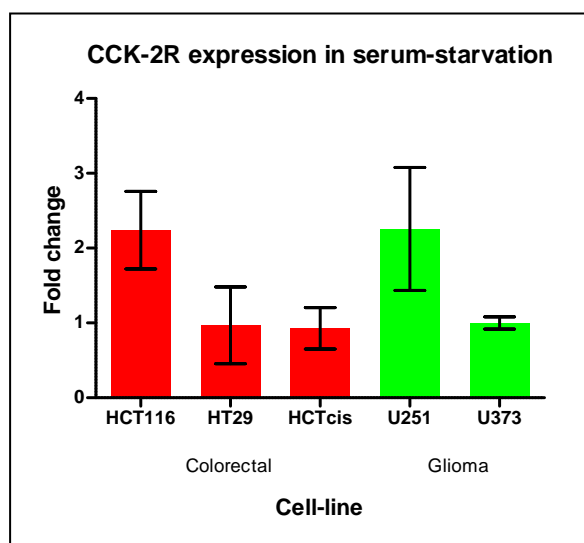
#### 4.1. The effect of microenvironment on CCK-2R expression

The mass of cells making up a tumour form a microenvironment where the conditions are different to that in normal tissues. The fact that many cells are packed together, causes hypoxia and nutrient deficiency because, although angiogenesis is encouraged within the tumour, the blood supply cannot be as efficient as it would be in the normal situation [167]. Cellular stress is a hallmark of cancer, and the hypothesis was that CCK-2R is up-regulated in these conditions. For example, Ashurst *et al.* found that nutrient-deprived cells showed an increase in CCK-2R expression [114].

Initially this chapter will investigate the effect of stress on the regulation of CCK-2R expression, and then in the second half transcriptional regulation by the CCK-2R promoter will be explored.

##### 4.1.1. Serum-starvation

The first cell stress condition investigated was nutrient deficiency. The cells were plated in serum-containing medium but once settled this was exchanged for serum-free medium and the cells incubated for 24 hours.



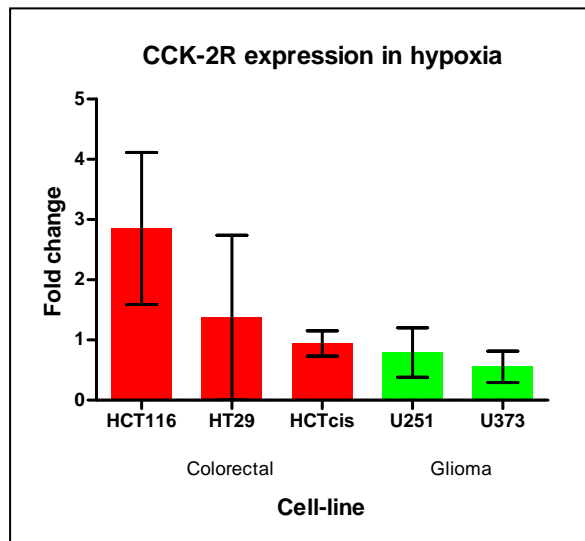
**Figure 4.1: Fold change in CCK-2R expression in serum-starvation**

*None of the colorectal or glioma cell-lines showed a significant difference between serum and serum-free conditions.*

Figure 4.1 shows that no significant difference was seen between the two conditions, although HCT116 cells show a trend towards up-regulation of the CCK-2R gene in serum-starvation.

#### 4.1.2. Hypoxia

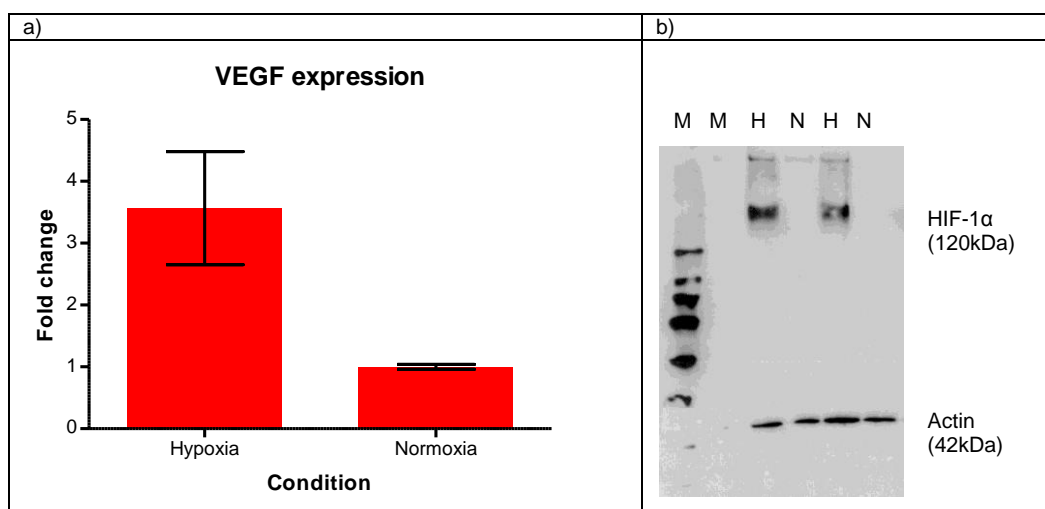
The cell-lines were also subjected to a hypoxic (1% oxygen) environment for 24 hours, however, this also failed to produce a significant change in CCK-2R gene expression (Figure 4.2). Again HCT116 cells showed a trend towards up-regulation of CCK-2R gene expression.



**Figure 4.2: Fold change in CCK-2R expression in hypoxia**

*None of the colorectal or glioma cell-lines showed a significant difference between normoxic and hypoxic conditions.*

In preliminary experiments VEGF gene expression measured by qRT-PCR and HIF-1 $\alpha$  protein expression measured by western blotting were used as verification of hypoxic stress in HCT116 cells (Figure 4.3). Since VEGF is 3-fold higher, and HIF-1 $\alpha$  only present in hypoxia the cells must be stressed, therefore this is not the reason behind the lack of significant up-regulation in Figure 4.2.



**Figure 4.3: VEGF and HIF-1 $\alpha$  expression in HCT116 cells in hypoxia**

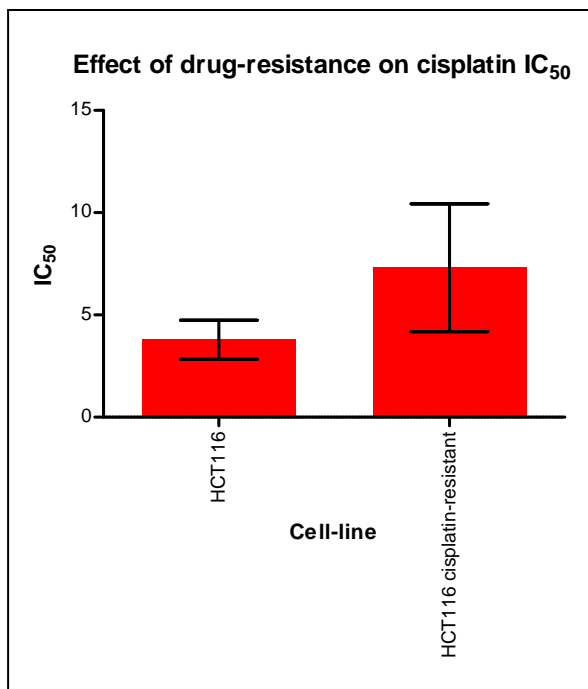
a) *qRT-PCR VEGF expression increases 3-fold in hypoxia compared to normoxia and*  
 b) *shows a western blot for HIF-1 $\alpha$  and actin for 2 hypoxic (H) and 2 normoxic (N) samples. M – marker.*

#### 4.1.3. Cytotoxic drug treatment

A cisplatin-resistant strain of HCT116 was developed, in order to discover whether this process would up-regulate endogenous CCK-2R expression, since the process would cause stress to the cells. To create these drug-resistant cells, wt HCT116 cells were grown in normal medium plus 0.01 $\mu$ g/ml cisplatin for three passages. The cisplatin concentration was doubled every three passages, assuming that the cells grew at a suitable speed and had not shown too much cell death. If this happened the cisplatin concentration was dropped or removed completely, to allow the cells to recover before starting to increase the concentration again. The cells were considered cisplatin-resistant when they had grown in medium containing 1mg/ml cisplatin for three passages.

To characterise these drug-resistant cells, an assessment of cisplatin IC<sub>50</sub> was carried out. This was compared to that of wild-type HCT116 in order to discover whether the IC<sub>50</sub> had increased. Figure 4.4 shows that the IC<sub>50</sub> for both cell-lines was not significantly different. The cisplatin-resistant cells did incline towards a higher IC<sub>50</sub>, but the SEM was very

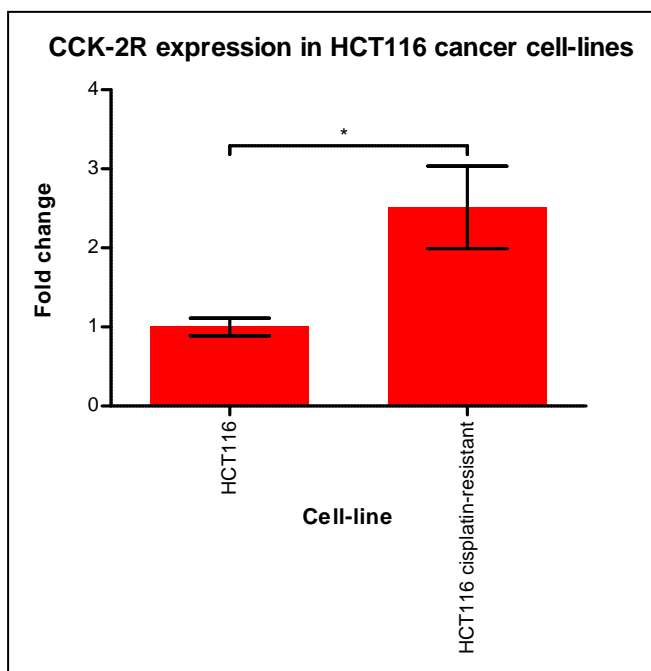
large, showing the large difference between the results of the replicate experiments.



**Figure 4.4: The IC<sub>50</sub> for cisplatin is measured for wild-type and cisplatin-resistant HCT116 cells**

*The method used is the MTT assay, and the result is an average of three repeats, with black bars representing the SEM.*

The two cell-lines, wild-type and cisplatin-resistant HCT116, were then tested in LATE-PCR for CCK-2R expression.

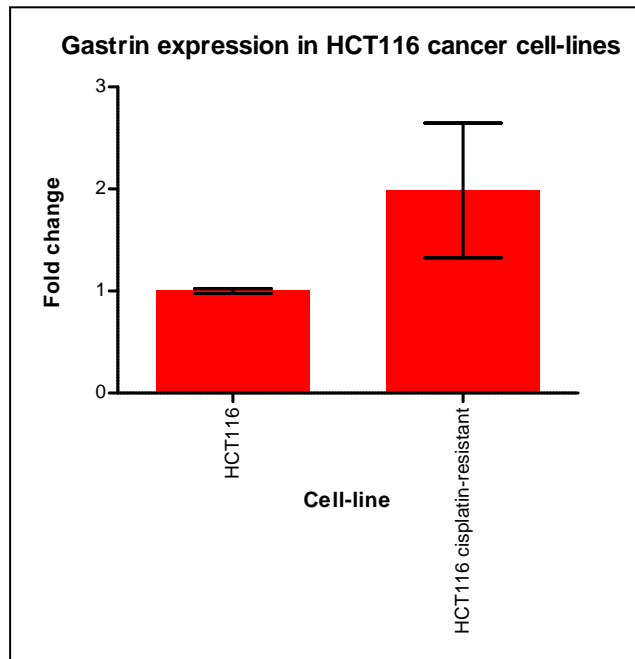


**Figure 4.5: Fold change CCK-2R expression in HCT116 cells; wild type and cisplatin-resistant**

*The method of detection of the CCK-2R is LATE-PCR, and the results are an average of four repeats with the black bars representing the SEM (\* indicates  $p < 0.05$ ).*

Figure 4.5 shows that CCK-2R was up-regulated in the cisplatin-resistant HCT116 cell-line, by almost three-fold ( $p < 0.05$ ). Gastrin gene

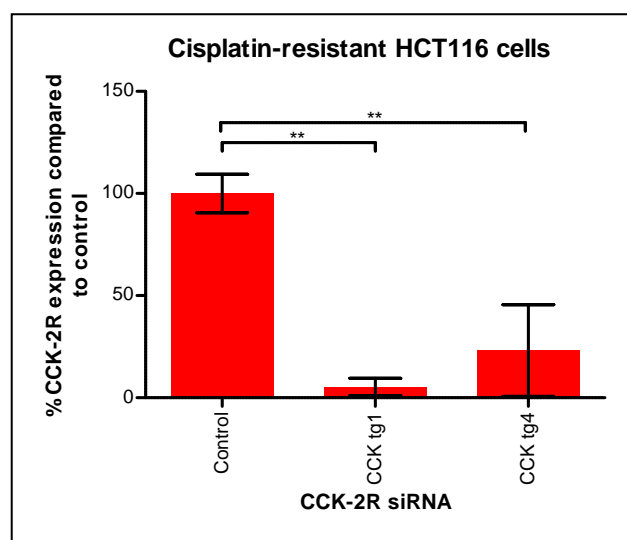
expression was also investigated in these two cell-lines but did not show a significant difference between them (Figure 4.6).



**Figure 4.6: Fold change gastrin expression in HCT116 cells; wild type and cisplatin-resistant**

*The method of detection of gastrin is Sybr Green qRT-PCR, and the results are an average of at least three repeats with the black bars representing the SEM.*

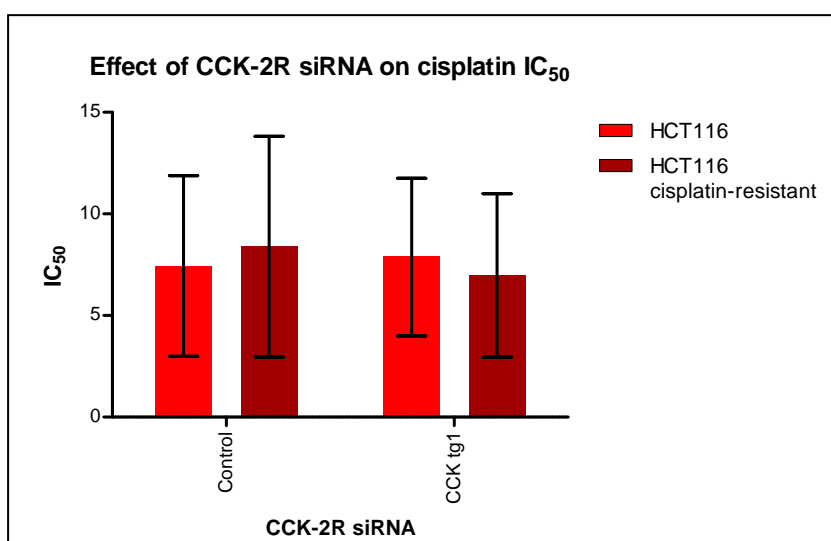
The CCK-2R expression shown in the drug resistant HCT116 cells can be knocked down by siRNA transfection; both CCK-2R siRNA had a significant effect on expression levels (CCK tg1 reduced the level to 5.3% and CCK tg4 to 23.1% of usual expression ( $p < 0.01$ ); Figure 4.7).



**Figure 4.7: Percentage expression of the CCK-2R in the cisplatin-resistant HCT116 cell-line when transfected CCK-2R siRNA**

*The method of detection of the CCK-2R is LATE-PCR, and the results are an average of two repeats with the black bars representing the SEM (\*\* indicates  $p < 0.01$ ).*

However, this siRNA knock-down does not affect cisplatin  $IC_{50}$  in either HCT116 cell-line (Figure 4.8), indicating that despite CCK-2R being up-regulated, it was not involved in the mechanism of drug-resistance.



**Figure 4.8:** The  $IC_{50}$  for cisplatin is measured for wild-type and cisplatin-resistant HCT116 cells transfected with a CCK-2R siRNA

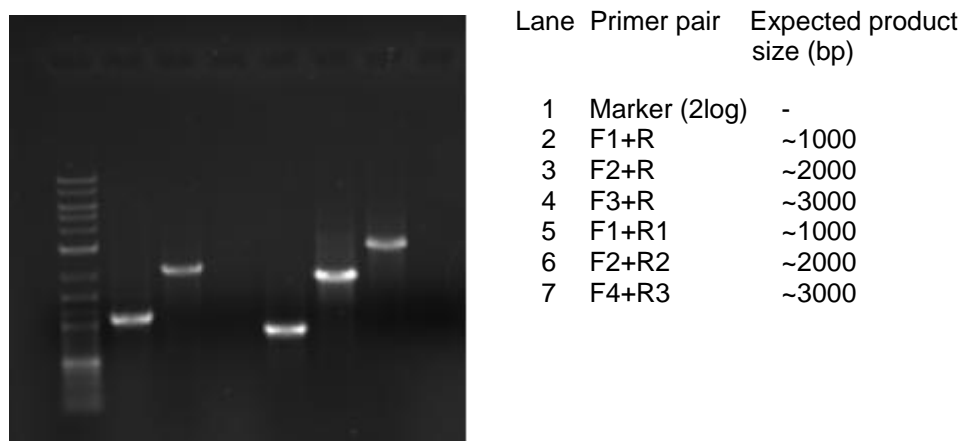
*The method used is the MTT assay, and the result is an average of three repeats, with black bars representing the SEM.*

## 4.2. Studies with the CCK-2R putative promoter

There is very little information in the literature on the regulation of the CCK-2R. In many cell-lines CCK-2R gene expression is low, while a number of biological effects of gastrin, via the CCK-2R, have been documented [13, 72, 74, 138, 139]. This could be due to regulation of the CCK-2R, or an indirect cause. In the only paper on the CCK-2R promoter, Ashurst & colleagues found that serum-starvation increased promoter activity in their large CCK-2R constructs [114]. We have also investigated the activity of the CCK-2R promoter, using reporter assays as we postulate that the low gene expression may be due to transcriptional regulation.

Initially plasmids were produced containing the putative CCK-2R promoter. Primers were designed to amplify 5kb of DNA upstream of

the CCK-2R start codon (named F and R), and classical PCR was used to amplify this fragment using H209 gDNA as a template. Unfortunately this did not produce any amplified DNA, so new primers were created to amplify sections 1, 2 and 3kb upstream from the start codon (Figure 4.9).



**Figure 4.9: Agarose gel showing classical PCR products for each of the primer pairs**

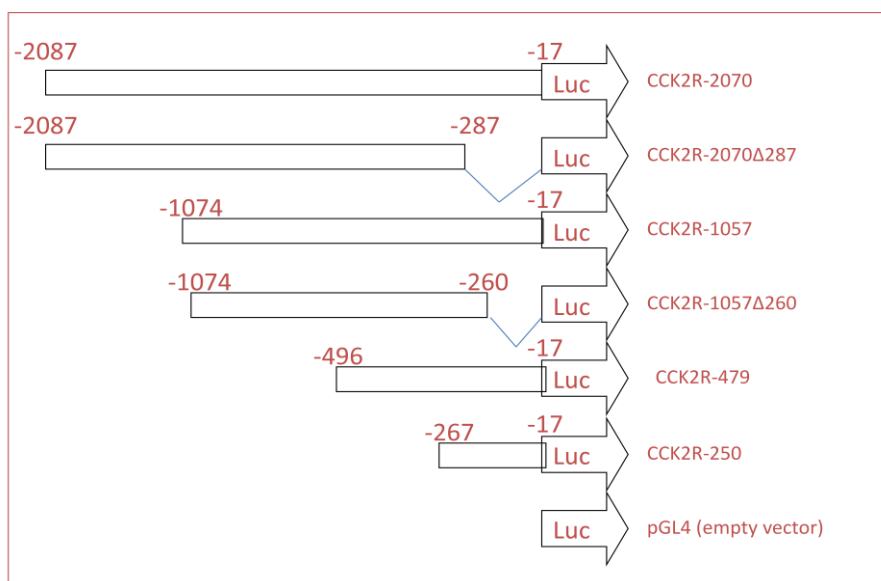
*The PCR was run with Q solution.*

The six PCR products (the four largest fragments are shown in Figure 4.9) were cloned into separate pCR®II-TOPO® plasmids, transformed and digested with *NcoI* and *XmaI* to determine their orientation (Figure 2.1). All six were then digested out of TOPO with *XhoI* and *SacI* (or *HindIII* if in the opposite orientation), and ligated into pGL4, which had been cut with the same restriction endonucleases (Figure 2.1). These constructs form a library of CCK-2R promoter fragments (Figure 4.10), ranging from 250-2000bp in size, including some deletion mutants which do not contain the first ~250bp of promoter sequence.

The pGL4 promoter constructs were given names to reflect the amount of the promoter present in each one. The largest construct was named CCK2R-2070, since it contained 2070bp of the sequence before the CCK-2R start codon (Figure 4.10). The second construct is labelled CCK2R-2070 $\Delta$ 287, because it contains the same DNA as the first one with 287bp of DNA proximal to the CCK-2R gene deleted. The rest are



named similarly, and contain increasingly shorter sections of the CCK-2R promoter.



**Figure 4.10: Diagram showing the relationship between the CCK-2R promoter constructs made during this project**

#### 4.2.1. Sequencing the CCK-2R promoter constructs

All six of the promoter constructs were sequenced, and had at least 97% homology to the start of the CCK-2R Ensembl [168] sequence. The differences between the sequences (highlighted in green in Figure A in the Appendix) are mainly restricted to just one construct, and are therefore possibly due to an artefact of the classical PCR. The large TC repeat section in particular shows several variations between the Ensembl sequence and that of the constructs, and this may be due to the inaccuracy of the sequencing reaction in those areas where there are large stretches of CT or CG repeats.

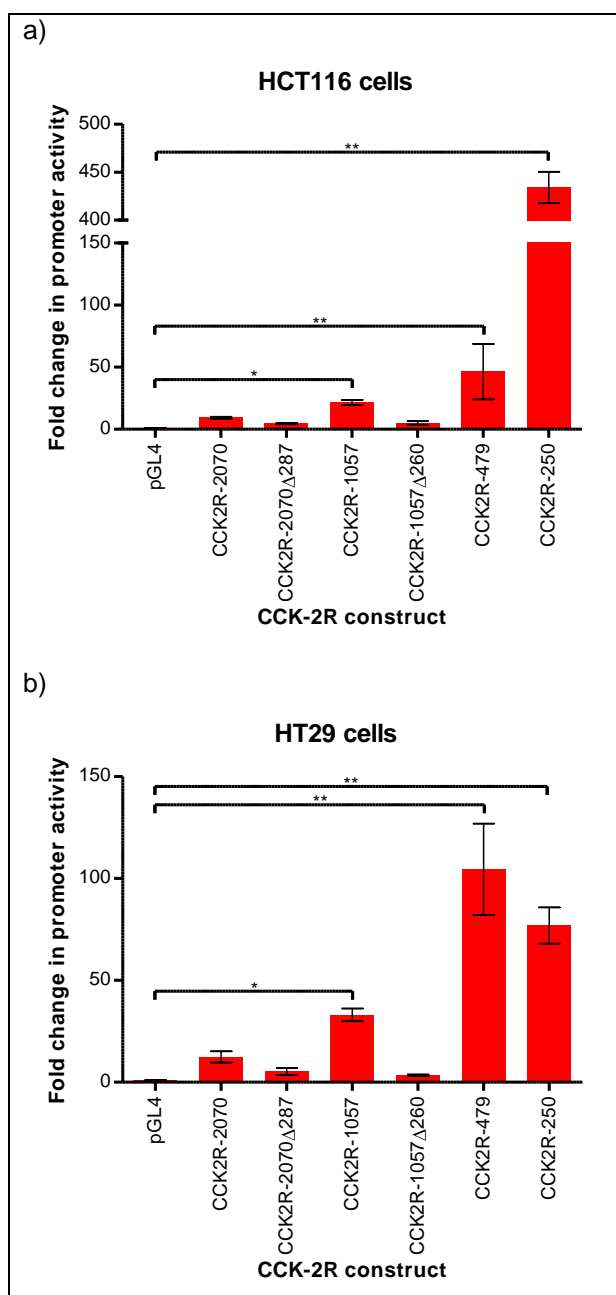
#### 4.2.2. Results with the CCK-2R promoter constructs

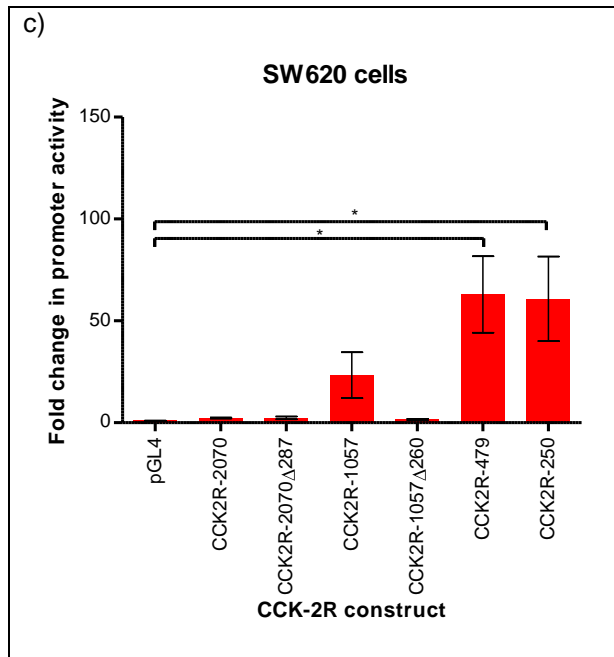
All six CCK-2R promoter constructs were transfected into a panel of cancer cell-lines, along with pGL4 as a negative control, to compare the

activity of the promoter in these cells, using luciferase expression as a readout.  $\beta$ -galactosidase DNA was co-transfected simultaneously with each plasmid, in order to control for differences in transfection efficiency between wells. The results for each construct were also normalised using the activity seen in the empty vector control, in order that fold change in promoter activity could be shown.

Figure 4.11 shows the results in colorectal carcinoma cell-lines, HCT116, HT29 and SW620. These three cell-lines all showed significant promoter activity when transfected with the two smallest constructs, CCK2R-479 and CCK2R-250. However, this activity varied between cell-lines. HCT116 cells showed 46 times the amount of control activity with CCK2R-479 and a further ten-fold increase with CCK2R-250 ( $p < 0.005$ ). HT29 and SW620 cells showed similar activity with both these constructs;  $\sim 90$  ( $p < 0.005$ ) and 60 ( $p < 0.05$ ) times the activity of the control, respectively. In addition to this, HCT116 and HT29 cells showed 21 and 33 ( $p < 0.05$ ) times control activity with the CCK2R-1057 construct.

Both deletion constructs and the largest whole promoter construct, CCK2R-2070, showed no significant activity in all three cell-lines, leading to the conclusion that the proximal 500 bases are the most crucial to promoter activity. There may also be repressor elements upstream of this important point, causing the two larger constructs to have less activity than the two smallest ones.

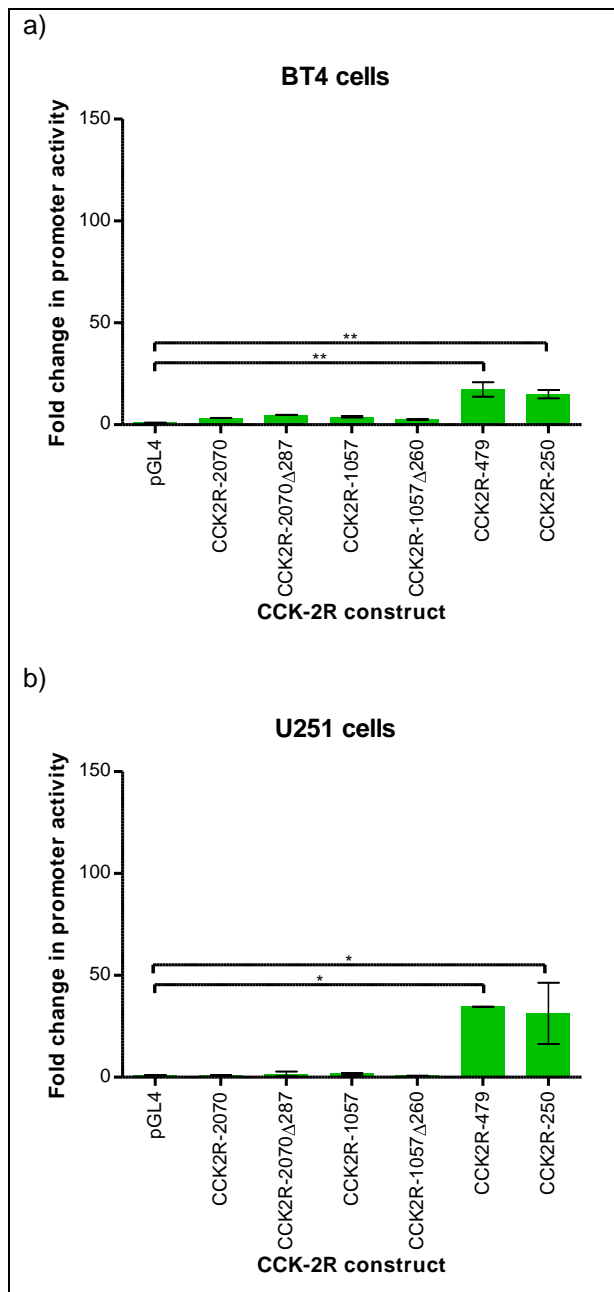


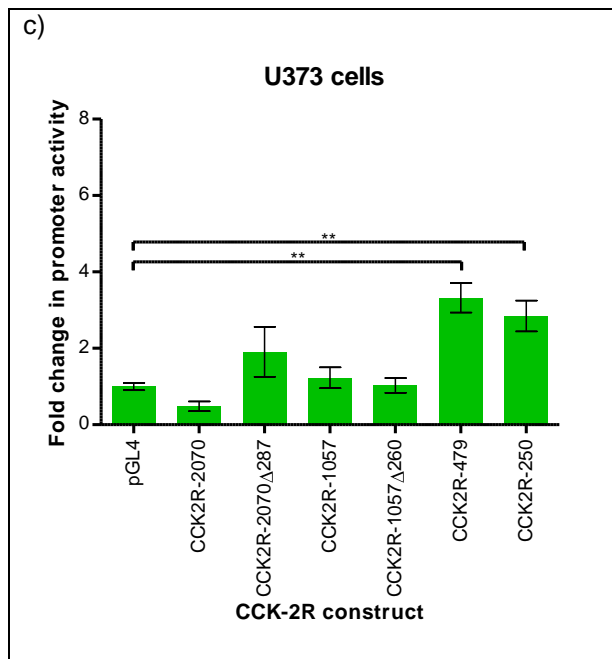


**Figure 4.11: CCK-2R promoter activity in 3 colorectal cell-lines**

a) HCT116, b) HT29 and c) SW620, measured using luciferase activity normalised by  $\beta$ -galactosidase activity. The results are also normalised to the activity seen in the empty vector, pGL4 (shown on the graphs with a value of 1). Each bar shows an average of four experiments, with the black error bars representing the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.005$ ).

Three glioma cell-lines were also transfected with the CCK-2R promoter constructs (Figure 4.12). BT4, U251 and U373 cells all showed significant promoter activity in the two smallest constructs; however these values vary considerably between cell-lines. BT4 and U251 promoter activity was 20-30 times ( $p < 0.05$ ) the control, whereas U373 activity was just 3 times ( $p < 0.005$ ) for both constructs. Interestingly CCK2R-1057, which was active in the colorectal lines, did not show significant activity in any glioma cell-line.

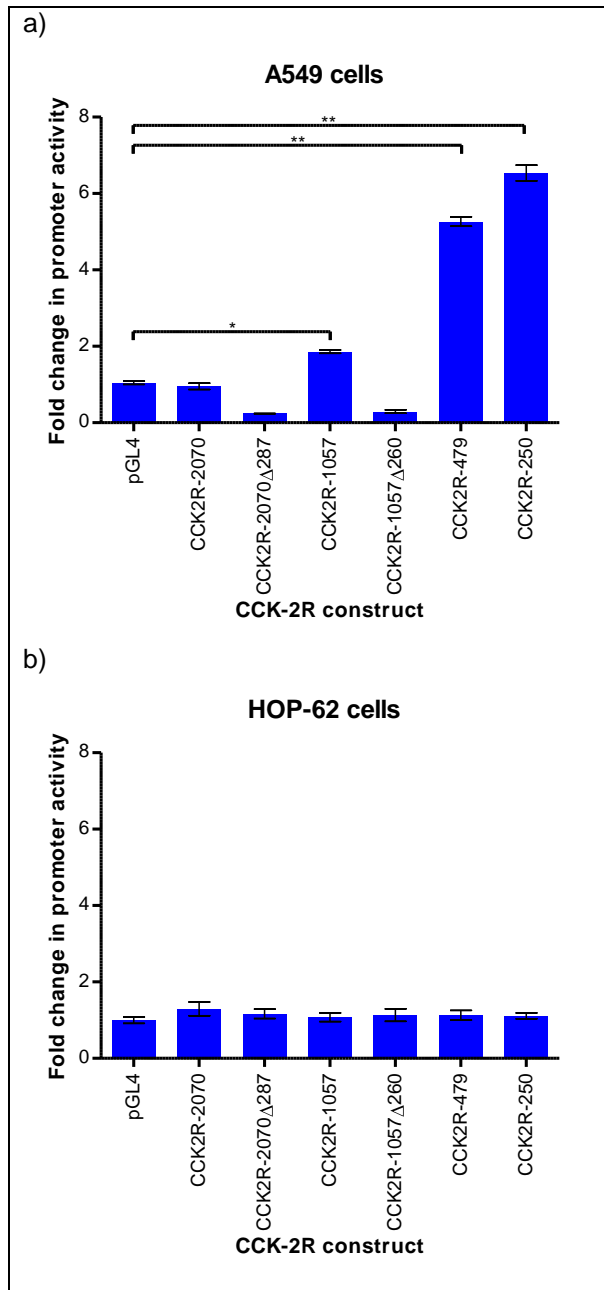




**Figure 4.12: CCK-2R promoter activity in 3 glioma cell-lines**

a) BT4, b) U251 and c) U373 (note the lower y-axis maximum), measured using luciferase activity normalised by  $\beta$ -galactosidase activity. The results are also normalised to the activity seen in the empty vector, pGL4 (shown on the graphs with a value of 1). Each bar shows an average of four experiments, with the black error bars representing the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.005$ ).

Two lung cell-lines were tested and showed very different results; the A549 cells demonstrated the same pattern as the glioma and colorectal cells, with significant activity in the 1, 0.5 and 0.25kb constructs ( $p < 0.05$ ), whereas HOP-62 cells showed no activity at all when transfected with any of the promoter plasmids, compared to the empty vector (Figure 4.13).



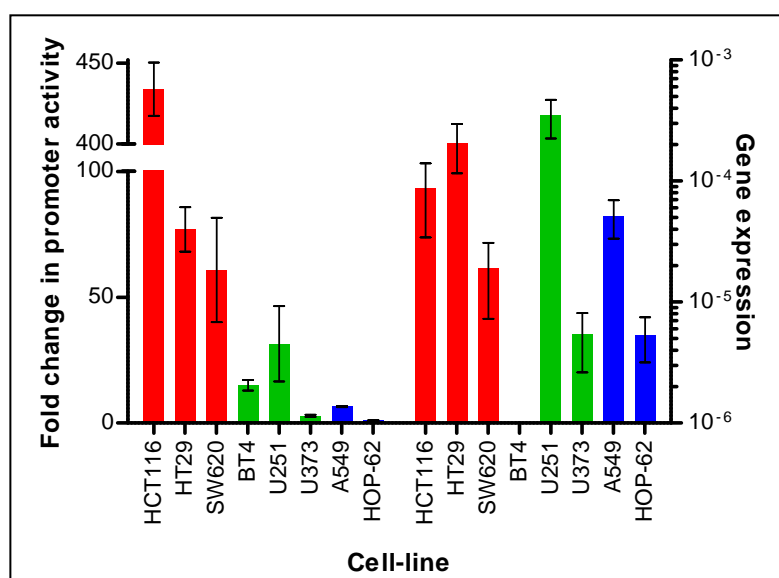
**Figure 4.13: CCK-2R promoter activity in 3 lung cell-lines**

a) A549 and b) HOP-62, measured using luciferase activity normalised by  $\beta$ -galactosidase activity. The results are also normalised to the activity seen in the empty vector, pGL4 (shown on the graphs with a value of 1). Each bar shows an average of four experiments, with the black error bars representing the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.005$ ).

### 4.3. Correlation of CCK-2R promoter activity with gene expression

CCK-2R gene expression varies considerably between cancer types and between cell-lines representing each cancer group. Figure 4.14 shows the gene expression levels (previously seen on a logarithmic

graph in Section 3.6) of the panel of cell-lines selected for the promoter studies, compared to the activity seen with the smallest promoter construct. Gene expression is shown on a linear, rather than logarithmic, scale in Figure 4.14 due to the smaller range in expression across this smaller panel of cell-lines. In Figure 4.14 it is clear that the colorectal cell-lines, U251 and A549 have higher levels of expression than U373 and HOP-62 cells. BT4 cells show no expression at all, representing a good control for the correlation with promoter activity.



**Figure 4.14: Comparing promoter activity to CCK-2R gene expression**

*Each experiment was carried out at least three times; promoter activity of the smallest construct, CCK-2R-250, (the black bars represent the SEM) on the left y-axis; data from Section 3.6 demonstrating the relative expression of CCK-2R ( $2^{-\Delta Ct}$ ) using LATE-PCR (the black bars represent the 95% confidence interval), on the right y-axis.*

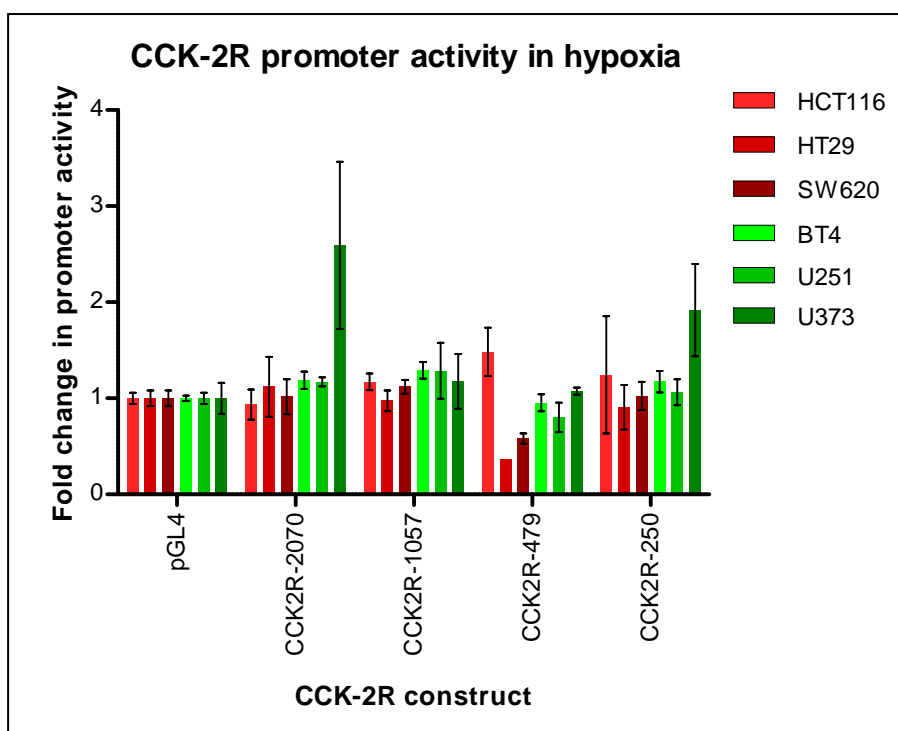
However, the pattern for promoter activity was different for CCK2R-250, which was the most active promoter construct; five of the eight cell-lines tested were within 30-90 times control activity (HT29, SW620, BT4 and U251 cells;  $p < 0.05$ ). HCT116 cells showed very high promoter activity at 430-fold and A549 cells showed lower activity at ~6-fold ( $p < 0.005$ ). Over the cell-line panel this does not correlate with gene expression (linear regression;  $p = 0.82$ ) since BT4 cells show no endogenous expression of CCK-2R, but high promoter activity. However, U373 and HOP-62 cells do show low expression and low or no promoter activity at



all. CCK-2R protein expression is not related to promoter activity either, due to the fact that HOP-62 cells have similar protein levels to the other cell-lines and HCT116 cells do not have high protein.

#### 4.4. The effect of hypoxia on CCK-2R promoter activity

Cellular stress is a very important factor in cancer progression. Stress did not affect CCK-2R gene expression but since this is not necessarily correlated with promoter activity it was possible that the stress condition, hypoxia, would have an effect on the activity of the promoter.



**Figure 4.15: CCK-2R promoter activity in 3 colorectal (red bars) and 3 glioma (green bars) cell-lines in hypoxia**

*Hypoxic activity is compared to normoxic activity at 1. This is measured using luciferase activity normalised by  $\beta$ -galactosidase activity. The results are also normalised to the activity seen in the empty vector, pGL4. Each bar shows an average of four experiments, with the black error bars representing the SEM.*

To test this idea an experiment was carried out with the colorectal and glioma cell-lines shown in the promoter studies above. This involved placing the transfected cells in hypoxia (1% oxygen) for 16 hours (previously found to be an optimal time-frame for hypoxic stress; HIF

induction, in our laboratory), a day after transfection. Figure 4.15 illustrates that a hypoxic environment does not have a significant effect on CCK-2R promoter activity in any of the six cell-lines.

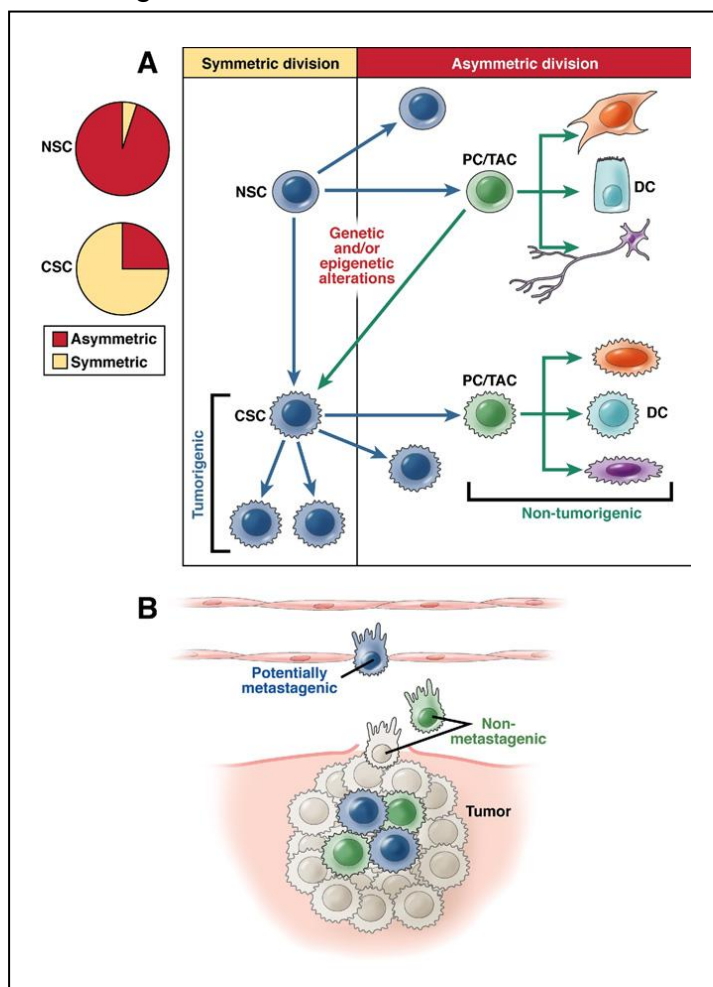
#### **4.5. Summary**

- CCK-2R gene expression was up-regulated in a drug-resistant colorectal cell-line, but not by stress in the form of serum-starvation or hypoxia.
- However, knock-down of CCK-2R did not affect cisplatin IC<sub>50</sub>, indicating that the receptor is not directly involved in the development of drug-resistance, but may be a surrogate marker.
- The 500bp region proximal to the start codon for the CCK-2R gene has been demonstrated to be active when transiently transfected into colorectal, glioma and lung cancer cell-lines.
- However, this activity is not correlated to gene or protein expression levels of the receptor.
- Our results show serum-starvation and hypoxia do not up-regulate promoter activity.

## Chapter 5 CCK-2R as a marker of cancer stem cells

## 5.1. Introduction

The environment in which a tumour cell lives has a major role in the development, survival and metastasis sites for that tumour [169]. In the normal stem cell niche, cell number is tightly regulated by balancing self-renewal with differentiation of daughter cells [170]. This leads to replenishment of the stem cell population but not the expansion which would lead to tumour growth. This regulation is achieved via paracrine signalling and feedback mechanisms [171]. However, these processes are deregulated when cancer cells form a cancer stem cell niche, allowing constant self-renewal without differentiation (Figure 5.1).



**Figure 5.1: Stem cell division**

*A normal stem cell (NSC) divides asymmetrically into one NSC and one progenitor cell (PC) or transit-amplifying cell (TAC) which will then produce differentiated cells (DC). If the NSC is genetically altered cancer stem cells (CSC) can be formed, allowing far more CSC to be produced in symmetric division. CSCs can also give rise to PC/TAC, which are non-tumourigenic. Image from [171].*

Cancer stem cells have been difficult to characterise due to contradictory studies demonstrating the relevance or irrelevance of specific markers or features, especially in colorectal cancer cells [33, 52].

As discussed in Chapter 1 many possible CSC markers have been described in the literature, but there is not yet a single marker or feature which defines the universal CSC. This makes it very difficult to choose a marker to measure cancer stemness, and so several have been used below, including CD133,  $\beta$ -catenin (as a marker of Wnt activity [172]) and CD44.

## 5.2. 3D cell culture

Many of the CSC markers described in Chapter 1 are highly expressed in a discrete population of cells [53], as seen in this project with the CCK-2R. In order to investigate whether these CCK-2R<sup>hi</sup> cells are a specific subset i.e. the cancer stem cell subset, cancer cell-lines were grown as 3D sphere structures. This method selects for stemness [45], meaning that if CCK-2R increased in these conditions, it would be linked to CSC formation or maintenance.

Growing cells in 3D culture; encouraging them to grow into spherical structures, is a much better model of the tumour environment, than growing 2D cell monolayers, since tumours themselves are three-dimensional complex structures [173]. In their review, Smalley *et al.*, state that cytoskeletal organisation, gene expression and the conditions of the microenvironment are significantly different in the same cells grown in 2D or 3D culture [174], and that 3D-cultured cells more closely mimic the *in vivo* situation [43, 45, 175]. In order to achieve a 3D culture of the cell-lines of interest in this project, the cells were re-suspended in 'stem cell medium', or SCM, a special low glucose DMEM mixed with

B27, F12-HAM and heparin, supplemented with EGF and FGF every three days.

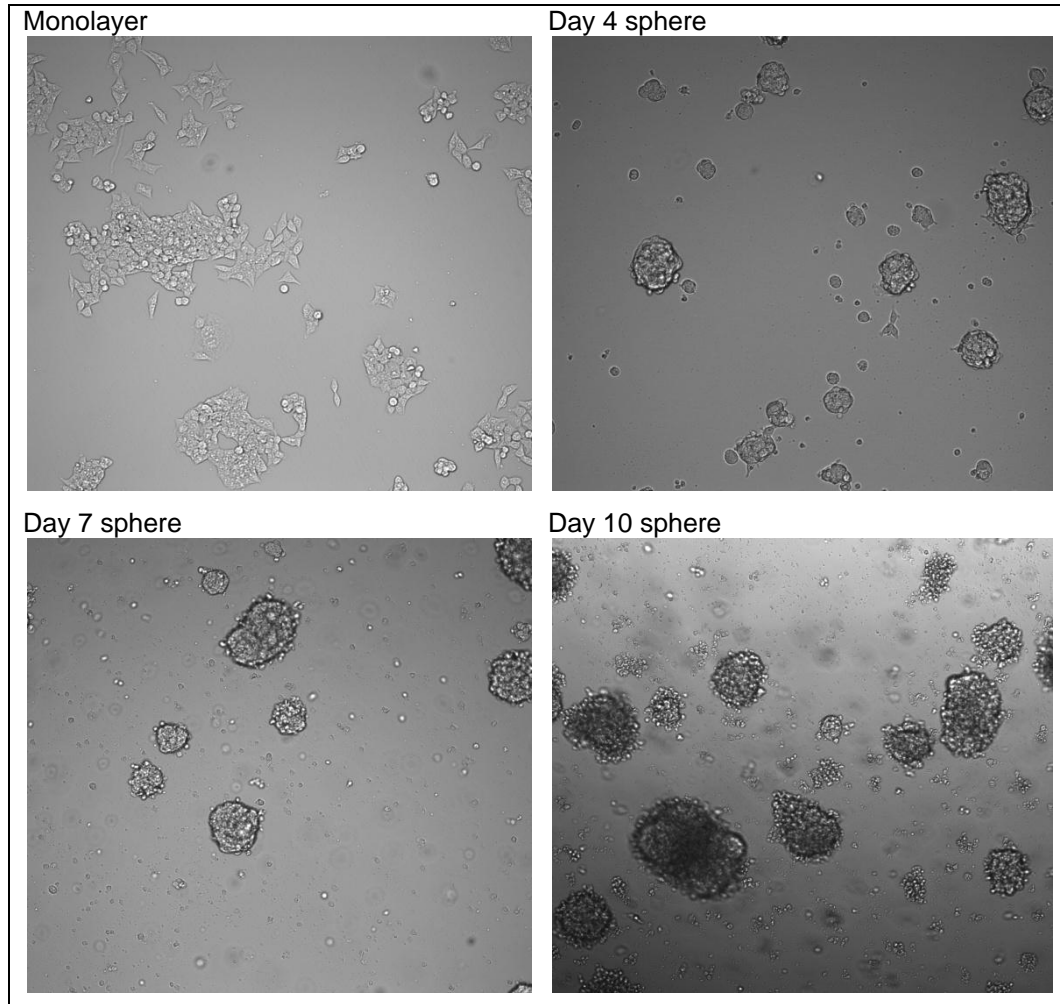
This is different to the spheroid structures being grown by other groups, where cell-lines were grown in agar or had a similar structure to support them, such as a basement membrane matrix [174].

The cells in this SCM grew into sphere-shaped structures, which looked very different to the flat monolayers usually seen in these cells (Figure 5.2). They often lifted from the culture plate and floated freely in the medium. The rate of formation, growth and the phenotype of the spheres differed across the cell-lines; however, all the colorectal, glioma and lung cell-lines tested formed 3D structures under the same conditions of nutrients and atmosphere (Figure 5.2). The colorectal cell-lines, HCT116 and HT29 grew into round structures made up of all the cells present in the culture. These then grew larger, becoming more defined at the edges as they grew, which is shown by the darker line around the larger spheres in the micrograph (Figure 5.2a & b). These structures were characterized as 'colospheres' [173]. The other colospheres, formed from SW620 cells, did not look the same, in that they were irregularly shaped and did not display a defined outer edge, so it was unclear whether these were colospheres or just clumps of cells. The cisplatin-resistant HCT116 cell-line produced similar colospheres to the wild-type HCT116 cells (Figure 5.2d).

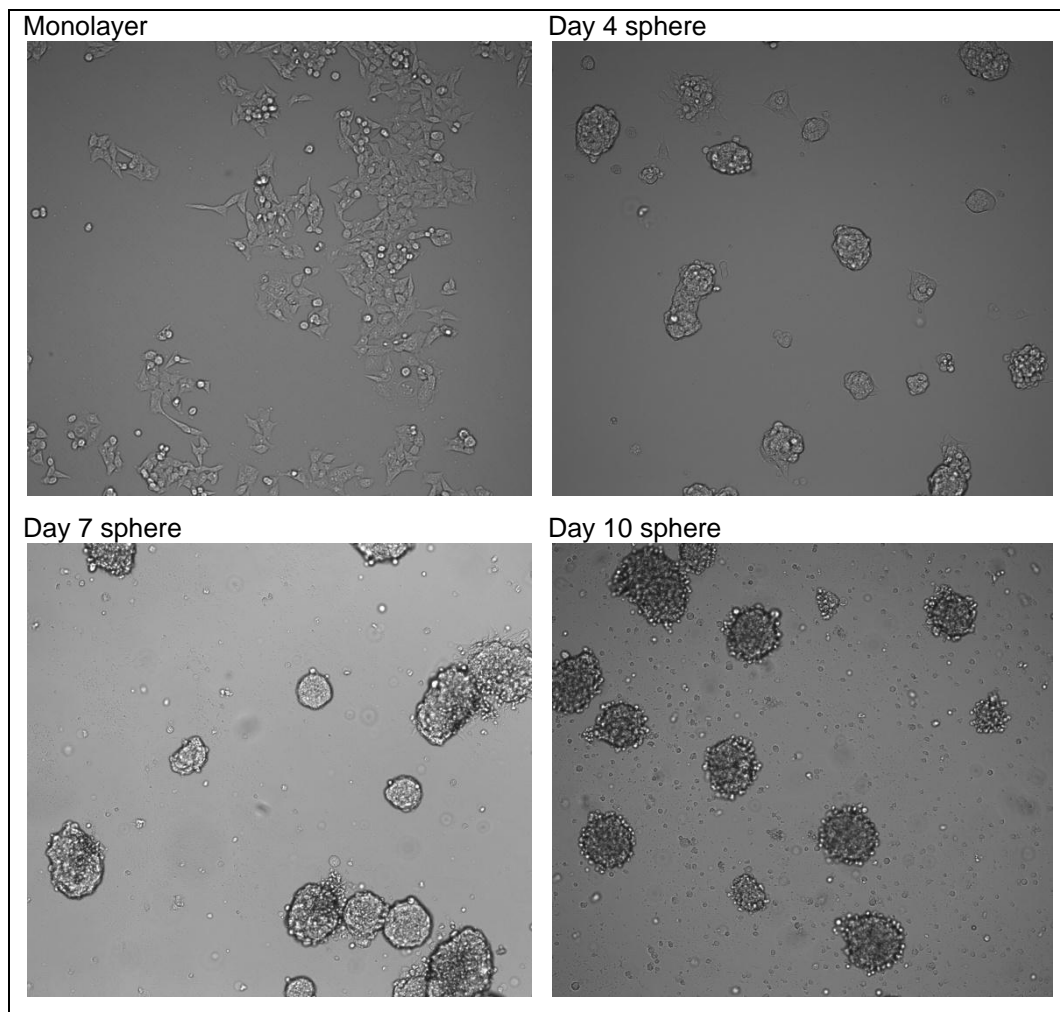
Neural cell-lines were the first cells to be grown as spheres, in the early 1990's, and were defined as neurospheres [176, 177]. The three glioma cell-lines used in this project formed neurospheres (Figure 5.2e, f & g), which looked quite different to the colospheres (Figure 5.2a, b, c & d), but fairly similar to the lung spheres grown (Figure 5.2h & i). The lung and neurosphere structures did not utilise all of the cells in culture

(some were left as monolayers), and although they formed clearly-defined masses, these had branches, unlike the colospheres.

a) HCT116

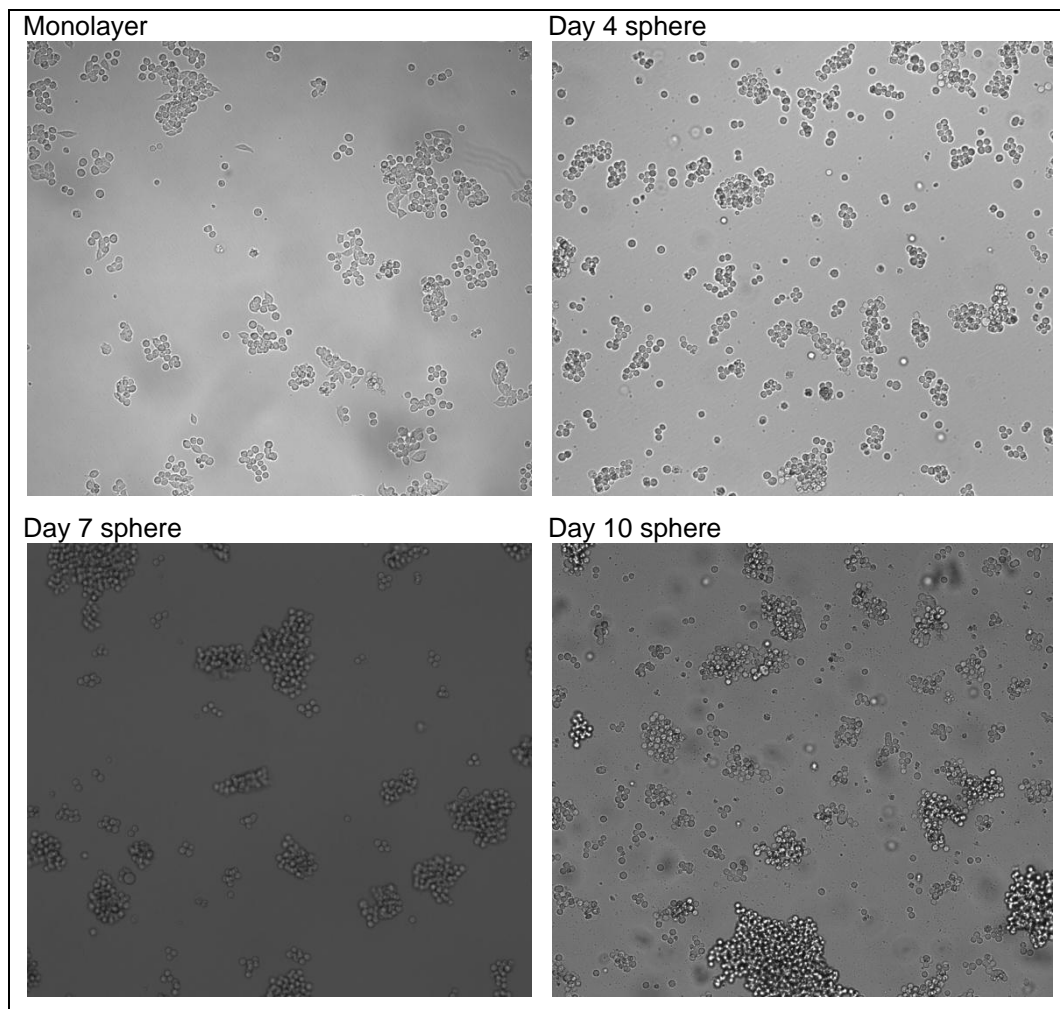


b) HT29

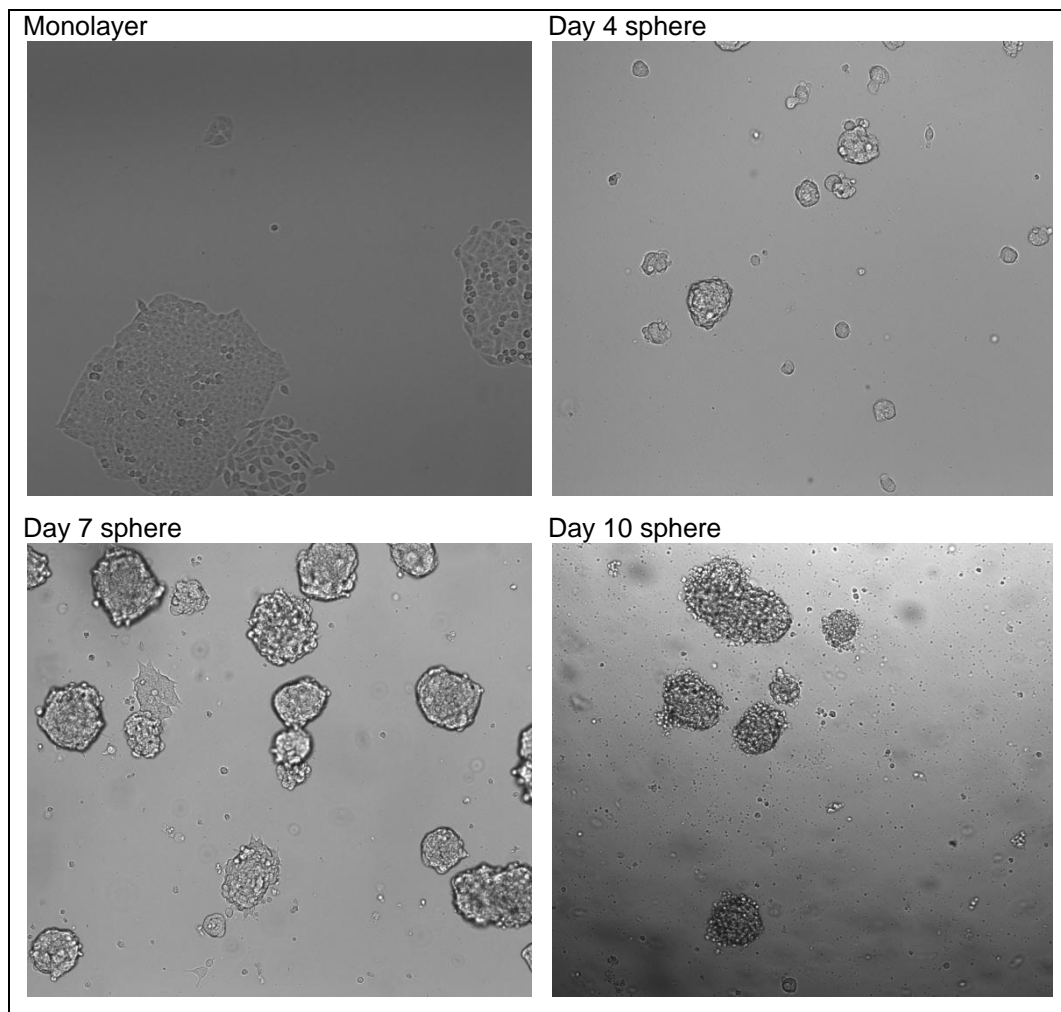




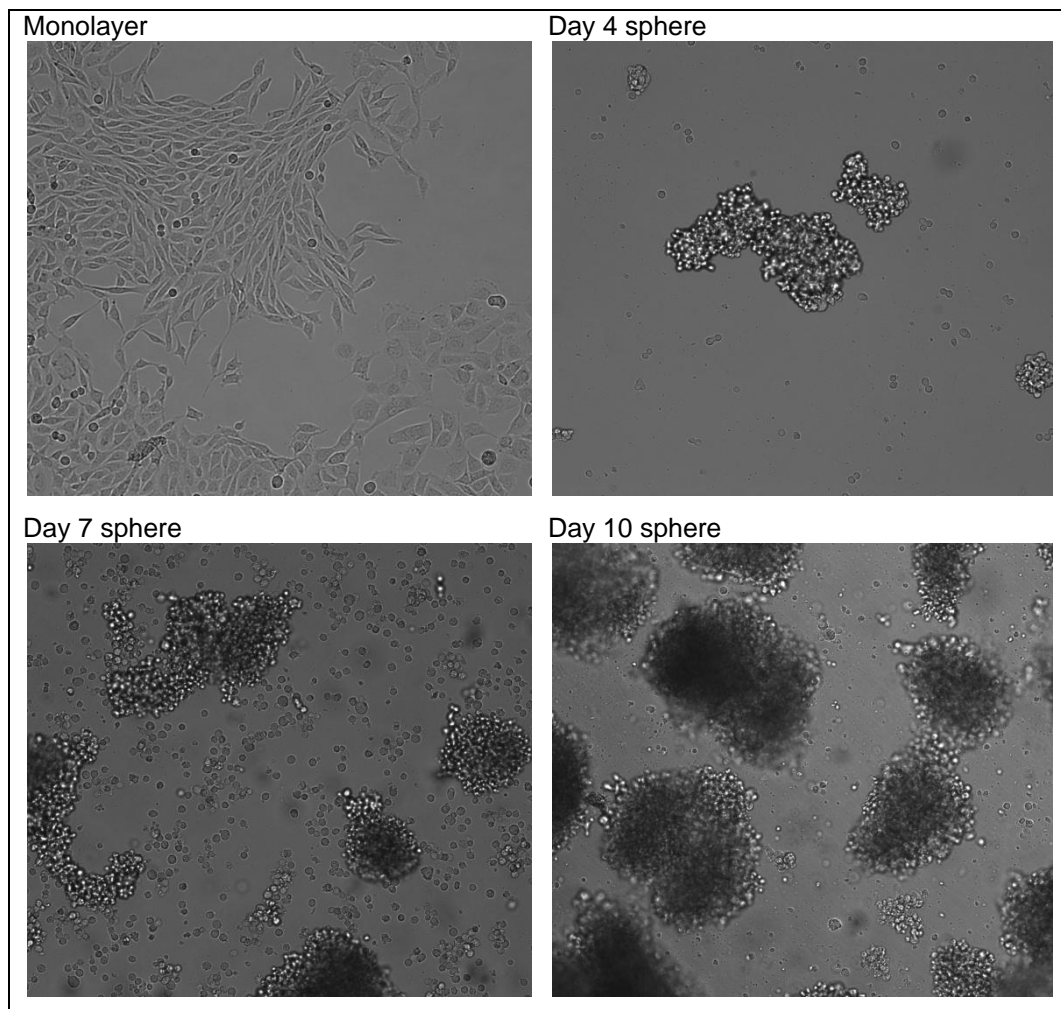
c) SW620



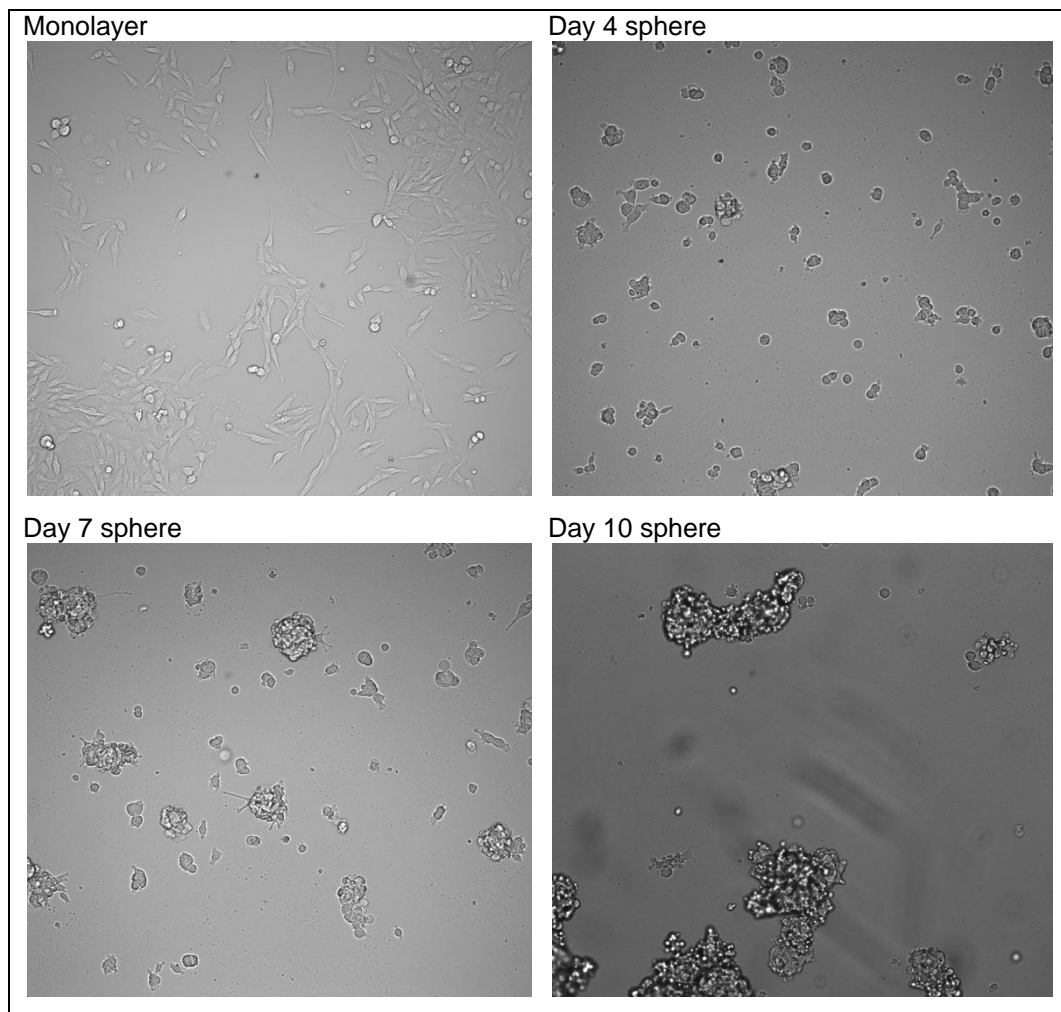
d) HCT116 cisplatin-resistant



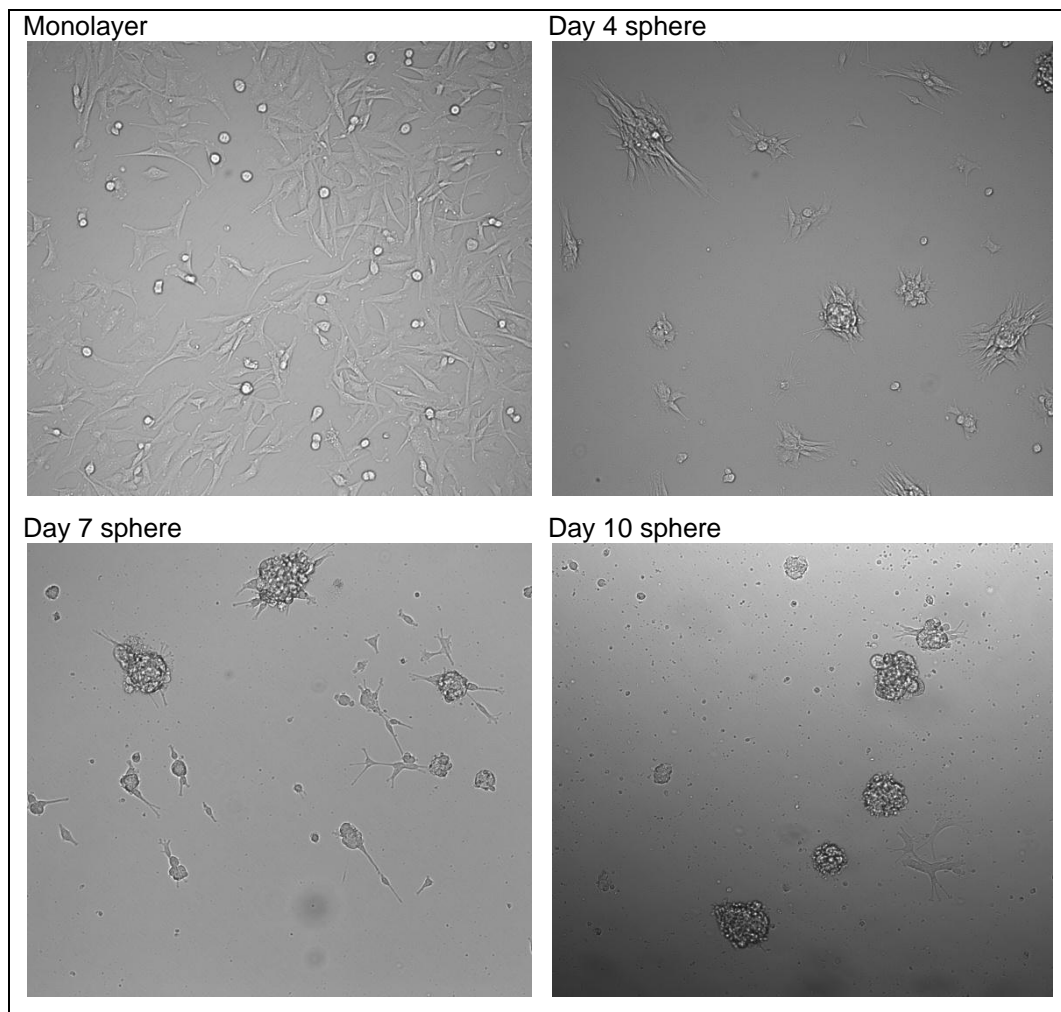
e) BT4



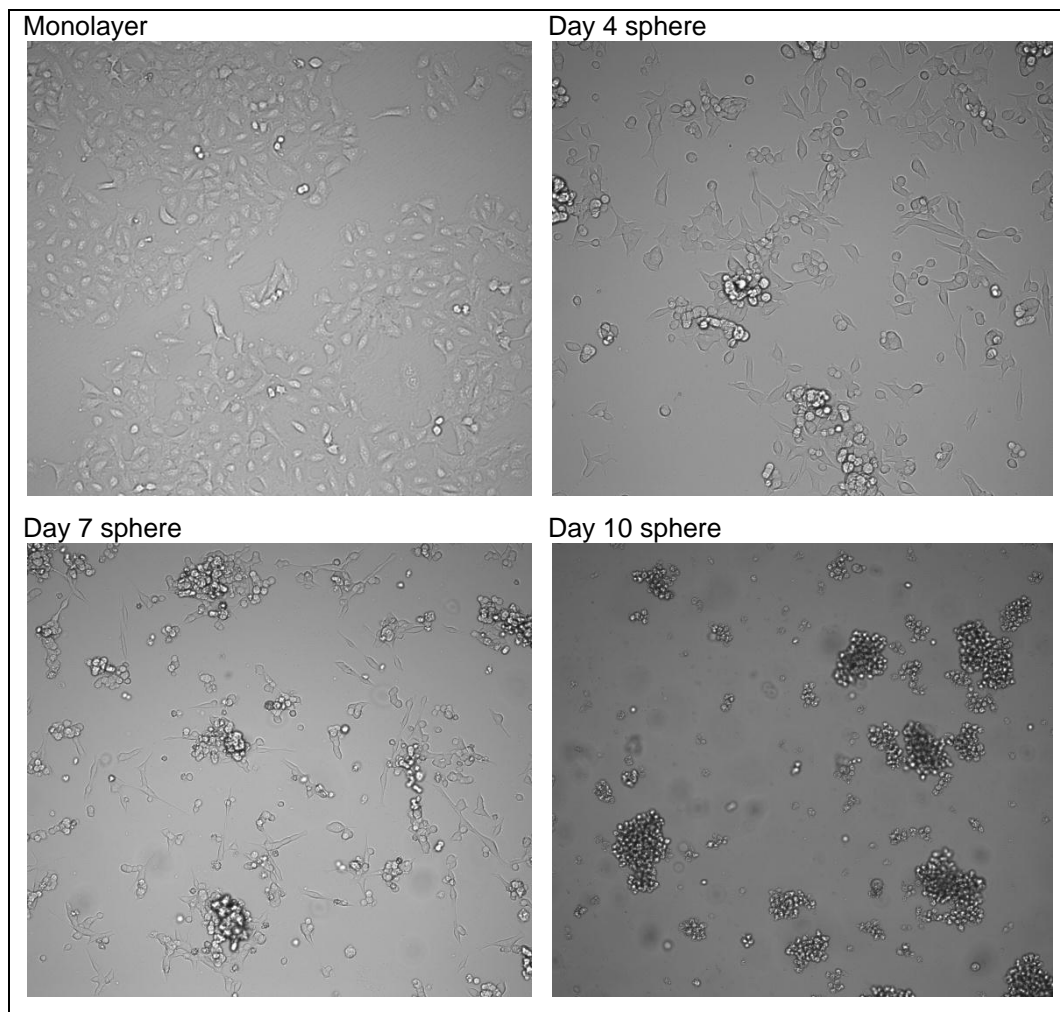
f) U251



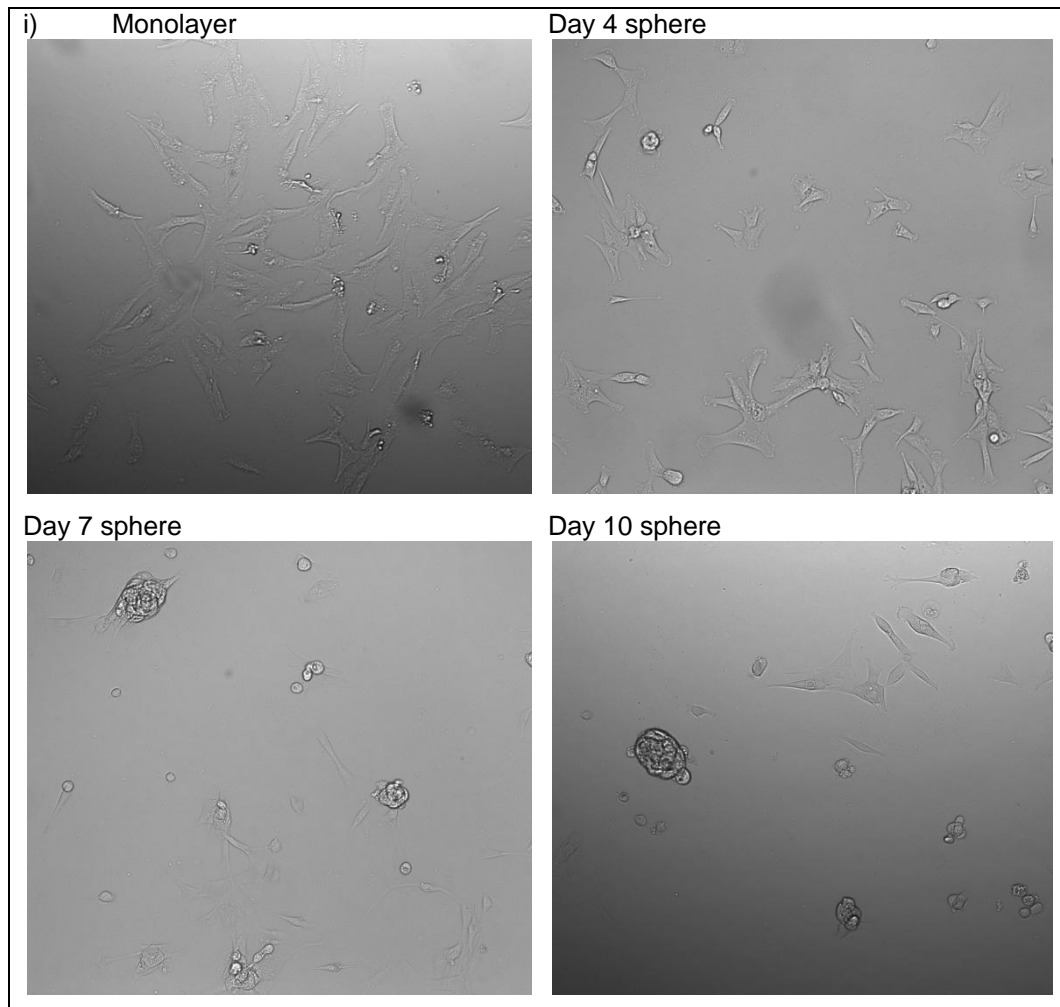
g) U373



h) A549



## i) HOP-62

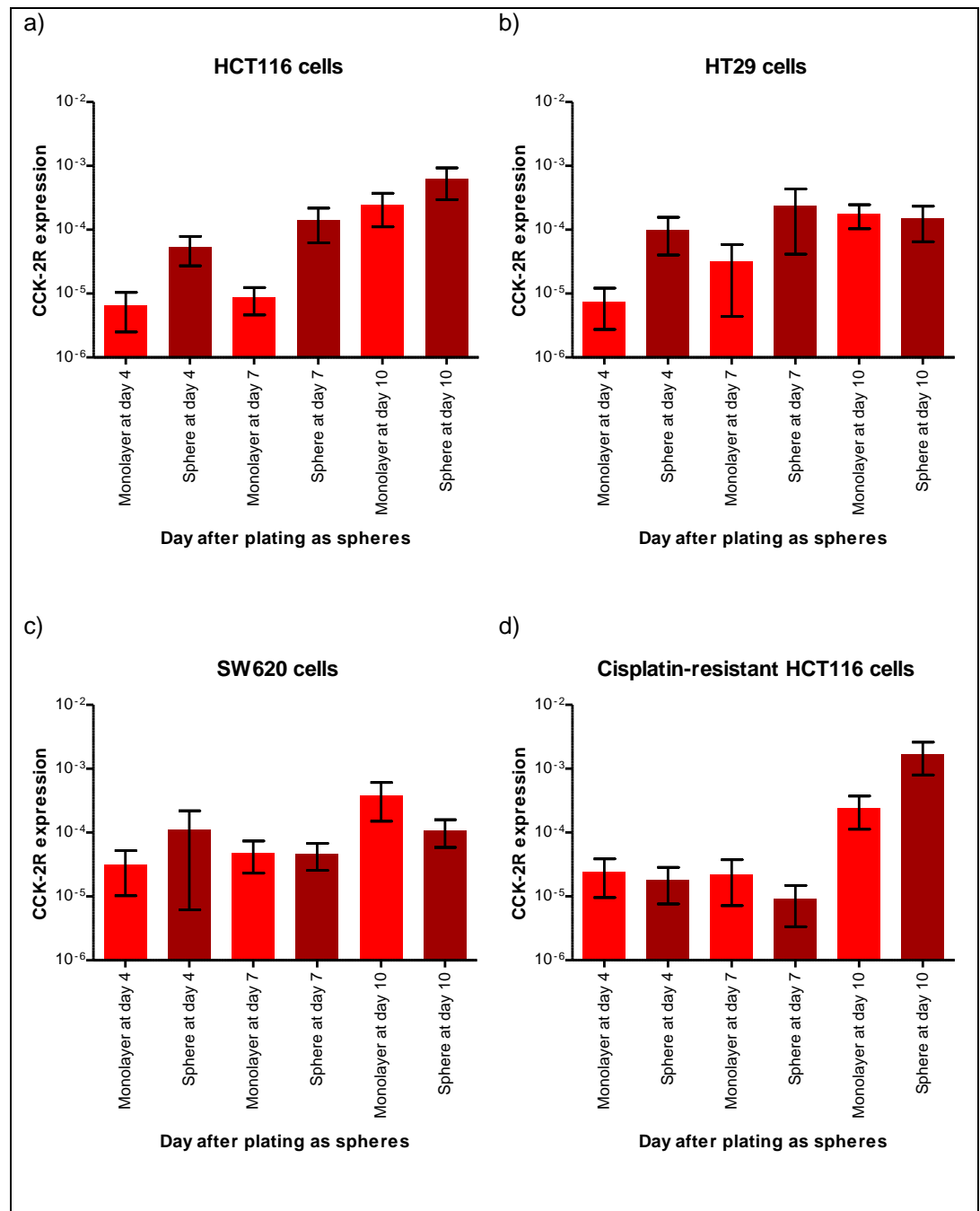


**Figure 5.2: Sphere structures formed from various cancer cell-lines**

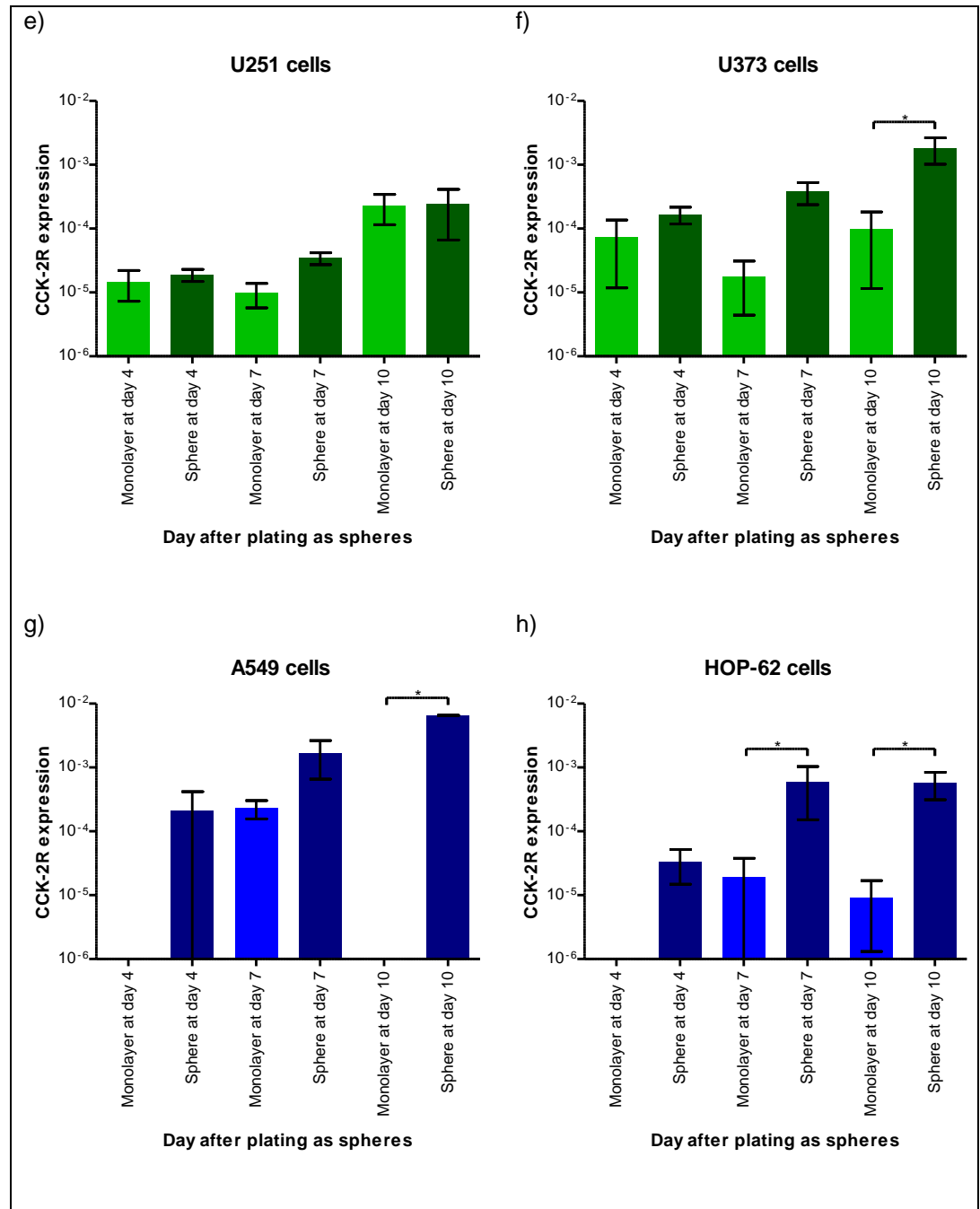
*Live photographs of a) HCT116, b) HT29, c) SW620, d) HCT116 cisplatin-resistant, e) BT4, f) U251, g) U373, h) A549 and i) HOP-62 cells; taken with the Nikon Eclipse Ti microscope at 10x magnification.*

### 5.3. CCK-2R expression in sphere cultures

CCK-2R gene expression was tested in the sphere-forming cells shown in Figure 5.2 compared to the same cell-line grown in monolayer culture. The day the cells were plated in SCM was taken as day one. A one-way ANOVA performed on the data from each cell-line showed significant up-regulation of expression in spheres compared to monolayers in U373, A549 and HOP-62 cells at day ten, and the latter at day seven as well (Figure 5.3).







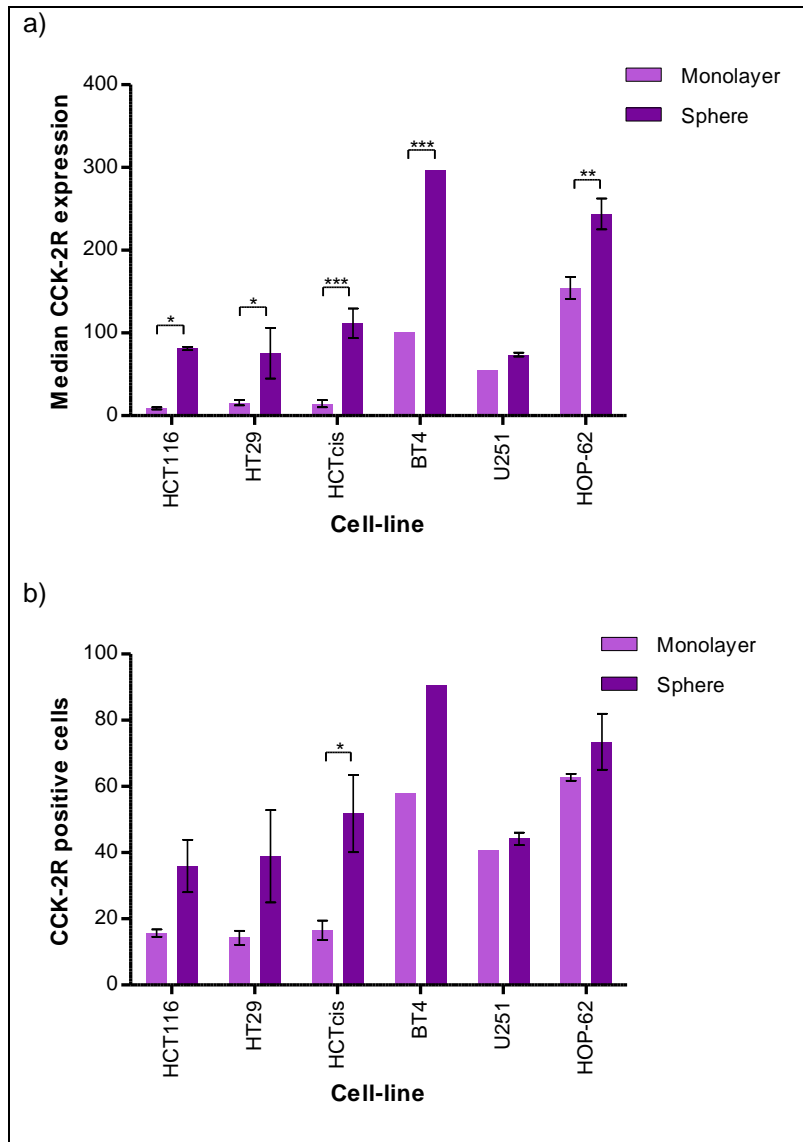
**Figure 5.3: CCK-2R gene expression in sphere-forming cells and those grown as a monolayer**

Logarithmic graphs showing CCK-2R gene expression in a) HCT116, b) HT29, c) SW620, d) HCT116 cisplatin-resistant, e) U251, f) U373, g) A549 and h) HOP-62 cells. BT4 cells (sphere and monolayer) did not show any expression of the receptor. The method of detection for the CCK-2R is LATE-PCR and the error bars represent the SEM from at least three replicates (\* indicates  $p < 0.05$ ).

No significant difference between the expression in spheres and monolayers was shown by this statistical test in the colorectal cell-lines and U251. However, the two-way ANOVA showed that CCK-2R expression increased significantly with time in HCT116, HCT116

cisplatin-resistant and U251 cells ( $p < 0.05$ ). It also correlated with the one-way ANOVA since CCK-2R expression increased significantly with sphere-forming in HOP-62 cells ( $p < 0.05$ ) and both parameters in U373 and A549 cells ( $p < 0.05$ ). The fact that HCT116 cells (wild-type and cisplatin-resistant) showed increased CCK-2R expression at day ten regardless of 2D or 3D culture may correspond with the trend for HCT116 cells to up-regulate CCK-2R in stress conditions (Section 4.1 above).

CCK-2R protein expression was also tested at day 10 of sphere-forming, in six of the cell-lines. SW620 cells were not included due to their sphere structures being very different and therefore not obviously 3D and the A549 cells grew very slowly so could not be included either. Protein expression was shown by two different methods; flow cytometry and paraffin staining, due to the fact that the former would show total CCK-2R protein expression in the sphere and monolayer cultures (Figure 5.4) and the latter the expression pattern through each sphere (Figure 5.5).

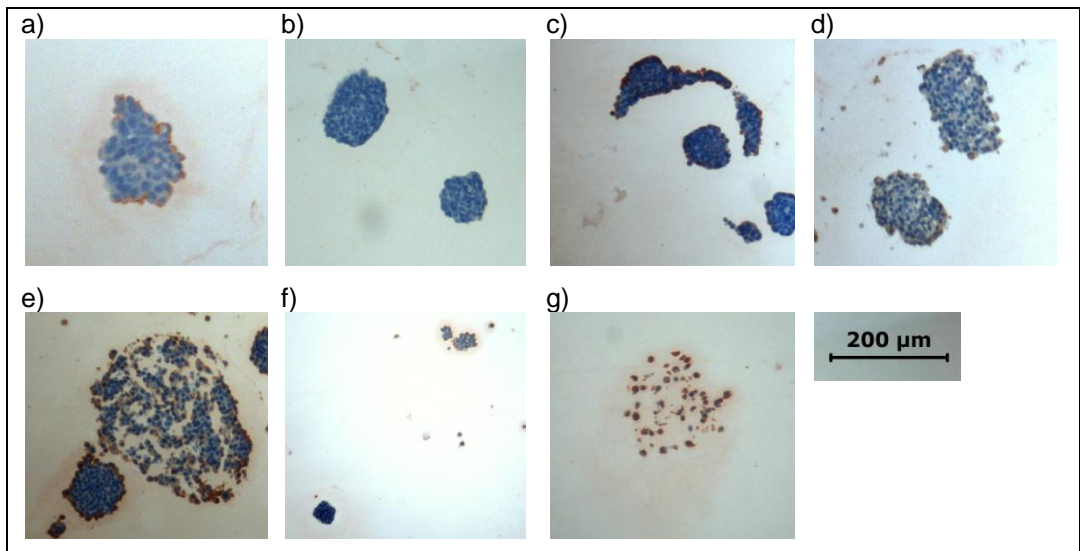


**Figure 5.4: CCK-2R protein expression in sphere and monolayer cultures shown by flow cytometry**

The method of detection for CCK-2R protein is live cell staining, followed by flow cytometry; shown by a) median FITC measurement and b) percentage of highly-positive cells. The negative samples (secondary antibody only) were used to normalise the data and the error bars represent the SEM from at least three replicates (except BT4 and U251 monolayer; \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.005$ ).

Live cell staining with an antibody to the second external loop of CCK-2R, followed by flow cytometry gave a measure of total CCK-2R expression in each population at day 10 (Figure 5.4). Figure 5.4 showed that a: expression of CCK-2R and b: the number of cells expressing a high level of CCK-2R, were very different values for each cell-line. The three colorectal cell-lines all showed significantly higher receptor expression in 3D culture ( $p < 0.05$ ); however, this was not true when the

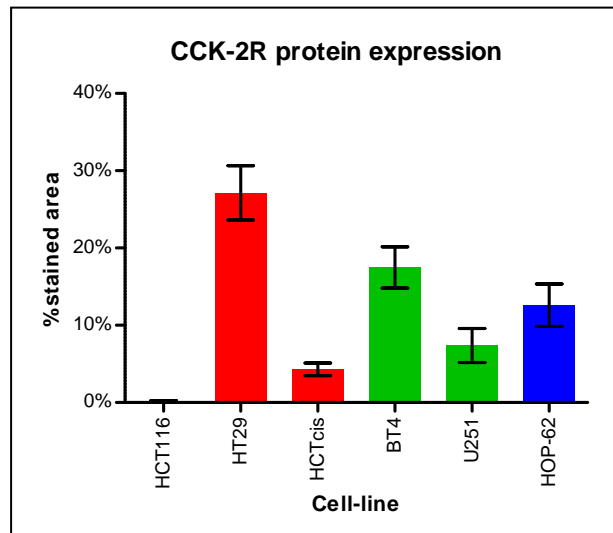
percentage of highly-positive cells was examined. BT4 and HOP-62 cells also displayed higher CCK-2R expression in sphere-forming cells, but U251 cells showed similar levels for 2D and 3D culture (Figure 5.4). This method gives no indication of where the positive cells are in the sphere, so in order to gain this insight; the spheres were embedded in paraffin wax and stained on slides (Figure 5.5).



**Figure 5.5: Paraffin staining for CCK-2R**

*Sphere structures were formed from cells and stained with a CCK-2R antibody (or the negative control with rabbit serum); a) HCT116, b) HCT116 negative, c) HT29, d) cisplatin-resistant HCT116, e) BT4, f) U251 and g) HOP-62. The scale of the photographs is shown by the marker for 200μm.*

Figure 5.5b shows an example of a negative control stained sphere. In the positive samples, the smaller spheres showed a higher percentage of CCK-2R staining than the larger spheres, and staining was most obvious in the cells around the outer edges of the sphere (Figure 5.5). This staining was analysed using QWin software, to find out the percentage staining across each sphere (Figure 5.6). HT29 and BT4 cells showed the highest percentage of staining (27 & 17% respectively), corroborated by the micrographs in Figure 5.5.



**Figure 5.6: CCK-2R protein expression in sphere cultures shown by staining analysis**

*The method of detection for CCK-2R protein is paraffin staining for a rabbit anti-CCK-2R antibody and the error bars represent the SEM from an average of all the stained spheres on each slide.*

In addition to CCK-2R, the sphere cultures were tested for various stem cell markers, such as CD133 and CD44, and also the ligand gastrin to see whether these were up-regulated with the receptor.

#### 5.4. Gastrin expression in sphere cultures

Gastrin was up-regulated at the gene level in monolayer HCT116 cells on day 4 ( $p < 0.01$ ; Figure B in the Appendix); however, there was no significant difference between spheres and monolayer cells in other cell-lines. The two-way ANOVA test also shows that time and culture methods did not have a significant impact on gastrin gene expression.

Comparing the gastrin gene expression graphs to the CCK-2R equivalents, the colorectal cell-lines showed very similar trends, except the cisplatin-resistant HCT116 cells, where gastrin was uniform across the graph and CCK-2R was up-regulated at day 10 (in both 2D and 3D culture). BT4 cells did not show expression of CCK-2R by LATE-PCR (despite displaying CCK-2R protein expression), and gastrin expression seems to have decreased by day 10. U251 and A549 cells showed up-regulation of CCK-2R at day 10 ( $p < 0.05$ ), but not of gastrin. However, U373 cells showed up-regulation of both CCK-2R and gastrin mRNA on days 7 and 10, even if the gastrin expression was not significant. The

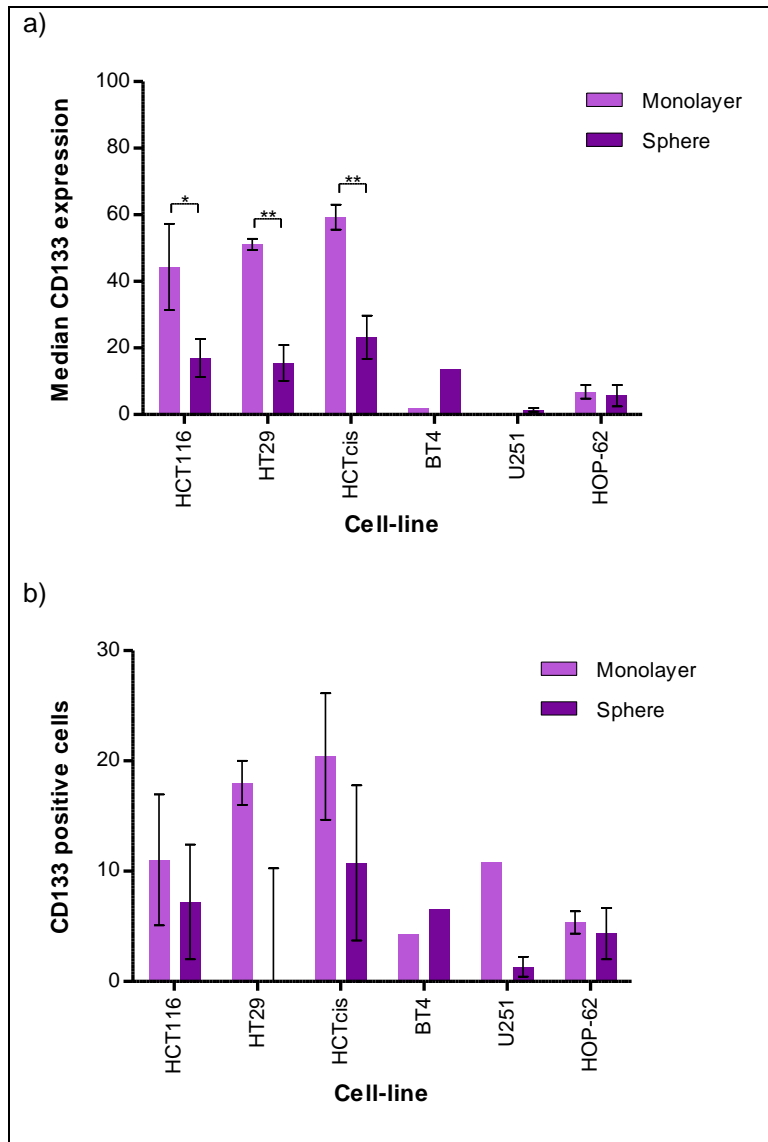
lung cells, HOP-62, actually showed the opposite effect in that gastrin decreased in spheres at day 7 and 10, in conflict with the CCK-2R expression.

### **5.5. CD133 expression in sphere cultures**

CD133, the most widely attributed cancer stem cell marker, was not significantly up-regulated in any of the cell-lines shown in Figure C in the Appendix. This was unexpected, especially in lung and neurospheres where CD133 is an accepted CSC marker, due to the fact that 3D culture should select for the cancer stem cells. However, these cells may differentiate after division, resulting in a similar CD133 expression profile to monolayer cells. The two-way ANOVA shows that time was a significant factor affecting CD133 expression in HCT116 and HCT116 cisplatin-resistant cells ( $p < 0.05$ ), and sphere-forming increased CD133 gene expression in HT29 and U251 cells ( $p < 0.05$ ). Otherwise the test showed no significance.

HT29 cells showed up-regulation of CD133 gene expression in spheres at days 7 and 10, but this was not similar to CCK-2R expression. As in the CCK-2R LATE-PCR, BT4 cells showed no expression of CD133 in qRT-PCR, despite exhibiting protein expression. The other glioma cells and lung cells showed uniform CD133 gene expression across sphere-forming/time, with no relation to the CCK-2R expression graphs.

CD133 protein expression in day 10 spheres was measured by flow cytometry (Figure 5.7). This marker was up-regulated in monolayers ( $p < 0.05$ ), compared to sphere-forming cells in colorectal cells, where CD133 is a contested CSC marker. BT4 cells showed a non-significant increase in CD133 protein expression in sphere-forming cells. U251 and HOP-62 cells showed no significant difference between the two populations.



**Figure 5.7: CD133 expression in sphere and monolayer cultures shown by flow cytometry**

The method of detection for CD133 protein is live cell staining, followed by flow cytometry; shown by a) median FITC measurement and b) percentage of highly-positive cells. The negative samples (mouse universal antibody) were used to normalise the data and the error bars represent the SEM from at least three replicates (except BT4 and U251 monolayer; \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).

The colorectal cells showed a negative relationship between CCK-2R and CD133 protein expression, and the other cell-lines demonstrated no correlation ( $p = 0.78$  for correlation of all medians).

## 5.6. $\beta$ -catenin expression in sphere cultures

Wnt signalling is often up-regulated in CSCs and this leads to the localisation of  $\beta$ -catenin protein to the nucleus, where it links to

transcription factors which activate target genes, such as c-myc and survivin [53, 172]. Figure D in the Appendix shows examples of spheres formed from different cell types stained for  $\beta$ -catenin.

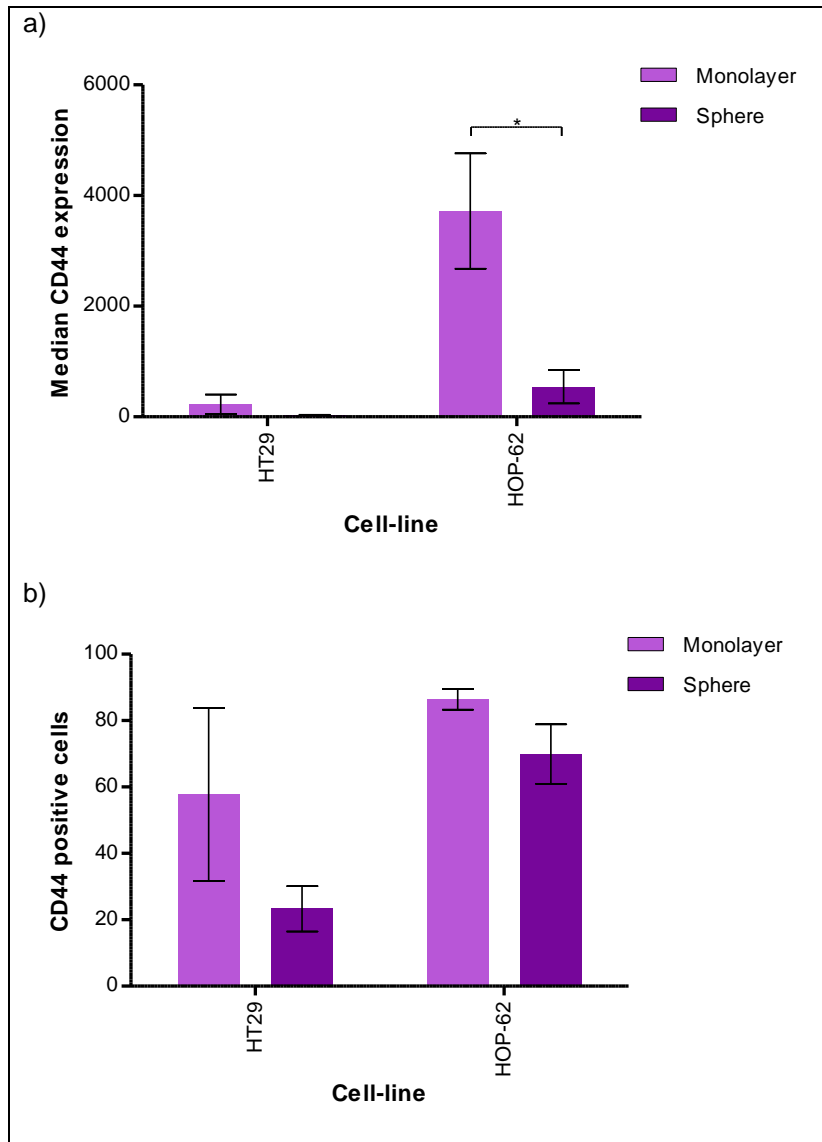
The staining shown for  $\beta$ -catenin was varied between spheres of a cell-line population, however, within each individual sphere it appeared to be uniform, and cytoplasmic not nuclear (Figure D in the Appendix).

### **5.7. CD44 expression in sphere cultures**

CD44 has been suggested to be a CSC marker in some cancers, e.g. colorectal carcinoma. This protein was detected by flow cytometry, using a conjugated primary antibody in live cell staining. This experiment was only completed on two cell-lines, HT29 and HOP-62, due to lack of time (Figure 5.8).

The median expression of CD44 protein showed that this is up-regulated in HOP-62 2D cultures, and not changed by sphere-forming in HT29 cells (Figure 5.8). These results supported the hypothesis that CD44 alone is not a CSC marker in colorectal or lung cancer. There was no relationship between CD44 and CCK-2R protein expression.



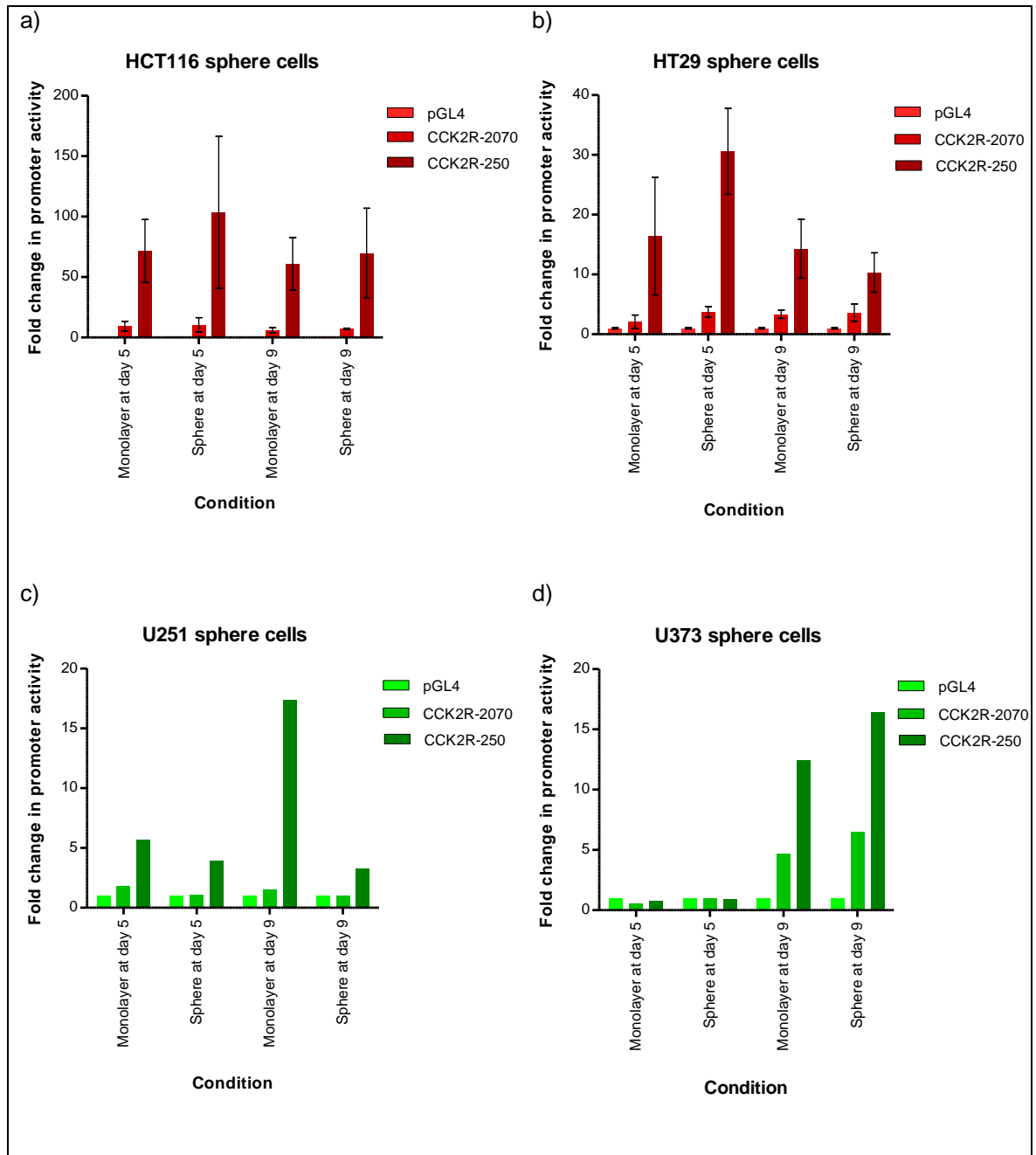


**Figure 5.8: CD44 expression in sphere and monolayer cultures shown by flow cytometry**

The method of detection for CD44 protein is live cell staining, followed by flow cytometry; shown by a) median FITC measurement and b) percentage of highly-positive cells. The negative samples (conjugated isotype antibody) were used to normalise the data and the error bars represent the SEM from two replicates (\* indicates  $p < 0.05$ ).

### 5.8. The effect of sphere-forming on CCK-2R promoter activity

Since CCK-2R expression is up-regulated in sphere-forming cells, the effect of this process on CCK-2R promoter activity was assessed. However, the cells show no significant difference in promoter activity between 3D and 2D culture (Figure 5.9).



**Figure 5.9: CCK-2R promoter activity in sphere-forming cells**

In two colorectal a) HCT116, b) HT29 and two glioma c) U251, d) U373 cell-lines, in sphere and monolayer culture. Promoter activity was measured using luciferase activity normalised by  $\beta$ -galactosidase activity. The results were also normalised to the activity seen in the empty vector, pGL4. In the colorectal graphs, each bar shows an average of three experiments, with the black error bars representing the SEM, but there were no significant differences between activity in sphere or monolayer culture.

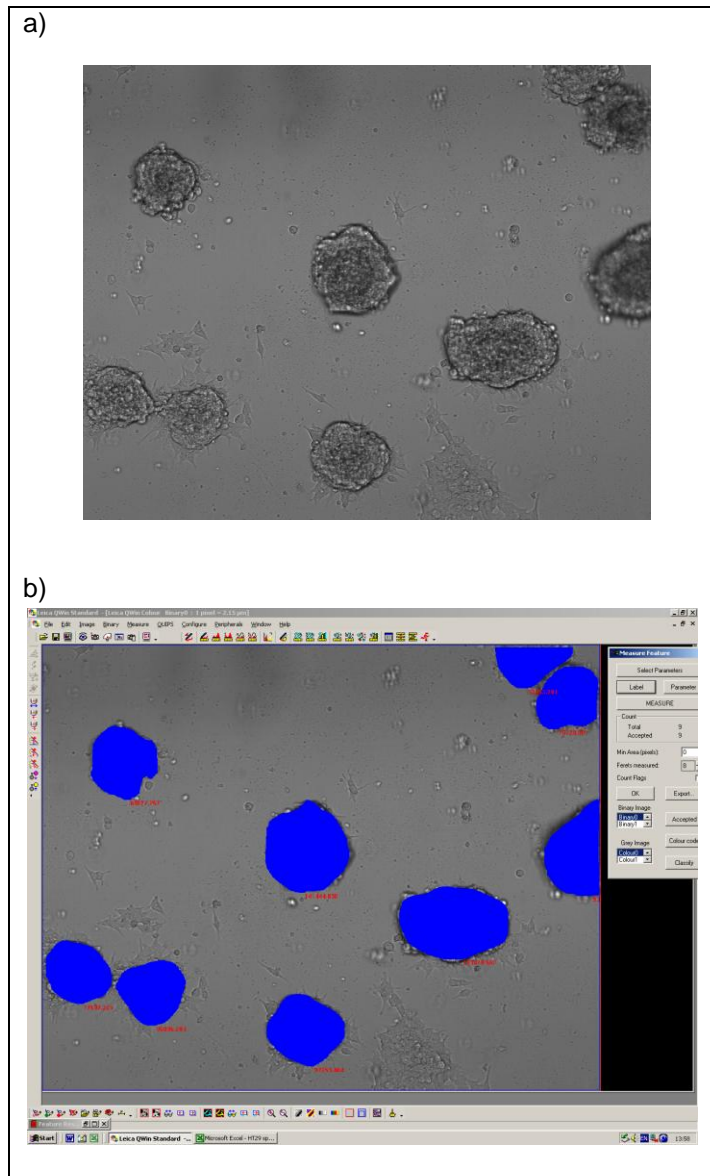
### 5.9. The effect of CCK-2R knock-down on sphere-formation

In order to investigate the functional role of CCK-2R the siRNA designed previously (CCK tg1) were used to knock-down the CCK-2R,

before forming spheres from these transfected cells. However, this was unsuccessful due to greatly reduced sphere-forming (by the CCK-2R and negative siRNA-transfected cells) and the concern that transient knock-down may not have been achieved for the whole ten days of sphere-forming, which was necessary to show an effect on CCK-2R gene expression.

A new experiment was devised using a CCK-2R antagonist to inhibit the receptor at the protein level instead, to prevent signalling through the receptor while sphere-forming took place. This was achieved using (R)-1-[2,3-Dihydro-1-(2'-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea, better known as YM022, a very selective CCK-2R inhibitor [178]. This experiment was carried out in five cell-lines, encompassing three cancer types, colorectal (Figure E in the Appendix), glioma and lung (Figure 5.11). The inhibitor was added at day 1, 4 and 7 in the YM022 group, at day 4 and 7 in the 'YM022 @ day 4' group and not at all in the untreated control group. The cells were photographed each day between day 2 and 10, but otherwise left in the incubator in 6-well plates at 37°C and 5% CO<sub>2</sub>.

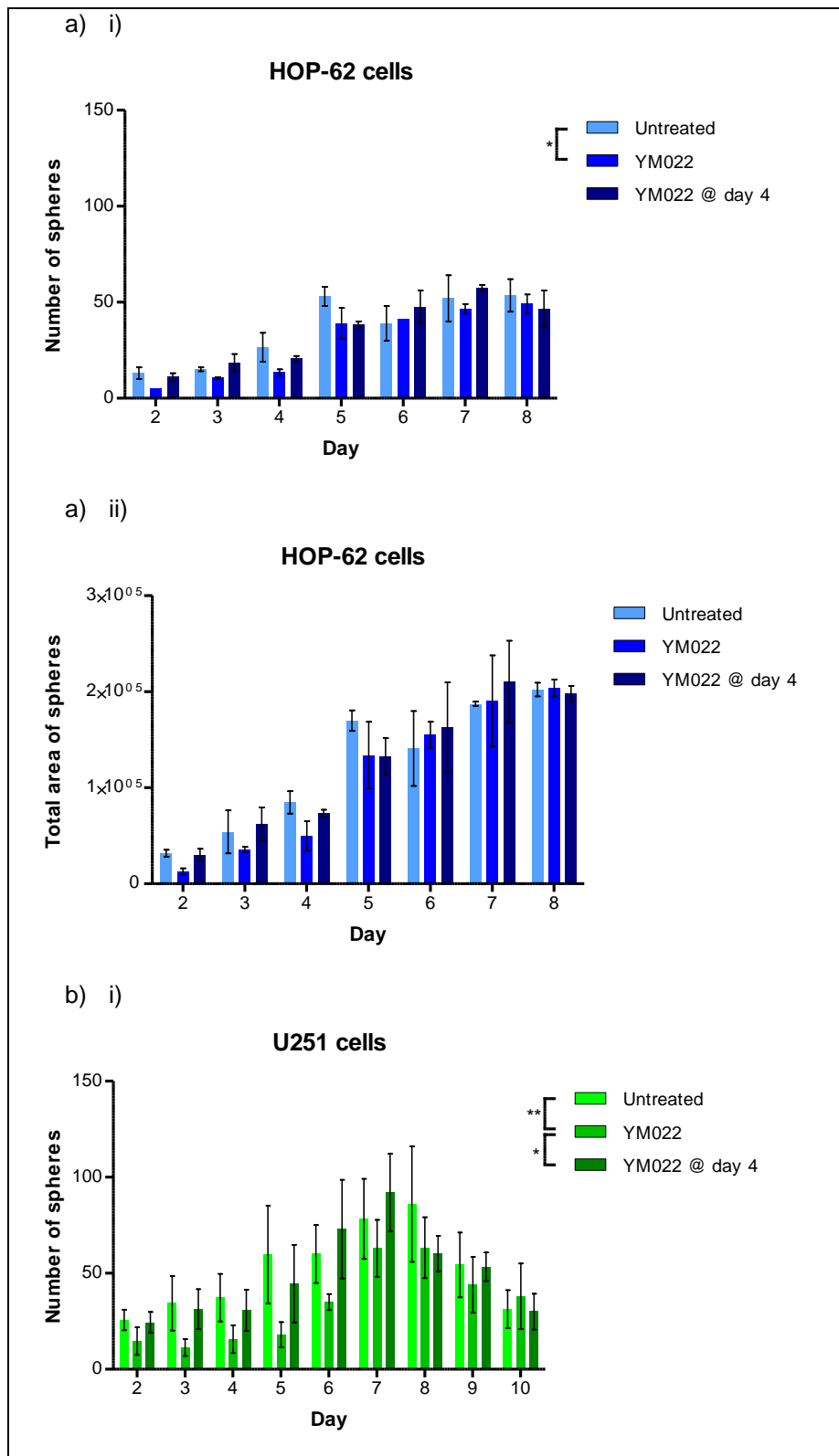
The staining analysis was carried out in QWin software for the Leica microscope. Each sphere in the photograph was highlighted, and then the software used to measure the area of each as shown in Figure 5.10.

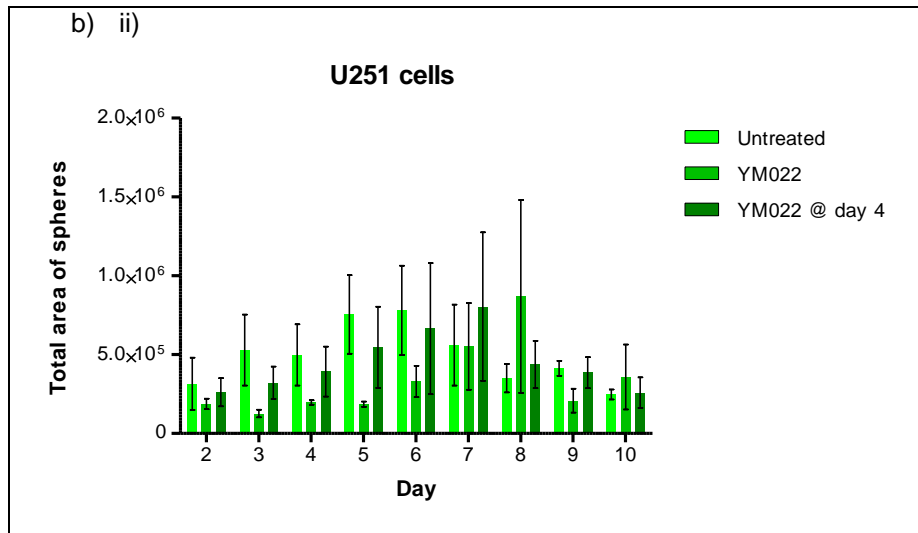


**Figure 5.10: An example of the analysis of sphere-forming**

In all the colorectal cells, number of spheres was low on day 2, and then stayed at a fairly constant number from day 3 onwards (Figure E in the Appendix). Sphere area gradually built up towards day 5, and then levelled out after day 6. Interestingly the HCT116 spheres were fewer in number than their cisplatin-resistant counterparts, but the sphere areas were slightly higher (in Figure E in the Appendix aii & cii the graphs have the same y-axis maximum ( $2 \times 10^6$ ) allowing direct comparison), showing that the resistant cells produced more, smaller, spheres. HT29 cells produced larger spheres (in Figure E in the Appendix bii the y-axis has a maximum of  $5 \times 10^6$ ) and more of them, possibly indicating a more

virulent cancer phenotype. Using the repeated measures one-way ANOVA statistical test, there was no significant difference between sphere number or area with the three treatments in any of the colorectal cell-lines.





**Figure 5.11: Total number and area of spheres formed each day in a glioma and a lung cell-line**

The cells were a) HOP-62 and b) U251 cells, with i) showing total number of spheres and ii) showing total area of spheres (note the different y-axis maximums). Data acquired from 10 photographs taken at 10x magnification for each condition on each day. These were also compared to their monolayer equivalents on each day. The bars show the mean of at least two experiments and the black error bars represent the SEM (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).

In Figure 5.11a ii the lung cell-line, HOP-62, was shown to produce smaller spheres than the other four cell-lines (y-axis maximum is only  $3 \times 10^5$ ). In both HOP-62 and U251 cells both number and area of spheres stayed low until day 4, with HOP-62 increasing at day 5 and then continued constant. U251 cells showed a more normal distribution with the peak of sphere-forming being day 7.

Using the repeated measures one-way ANOVA statistical test, addition of YM022 to the growth medium at day 1 caused HOP-62 sphere number to be significantly less than in the untreated cells ( $p < 0.05$ ). Sphere area was not significantly affected, but the graph in Figure 5.11a ii does show the same trend, with the YM022-treated cells being lower than the other two treatments until day 4. This indicated that inhibition of CCK-2R was reducing the cells capacity to form spheres between day 2 and day 4, when this capacity was restored to full measure. This restoration cannot be due to lack of inhibition by YM022, since it was replenished on day 4. The YM022 @ day 4 group, however

did not show significantly different results to either of the other treatments.

In U251 cells this effect was also shown, with the YM022 group showing significantly lower sphere numbers than the untreated cells ( $p < 0.01$ ) and the YM022 @ day 4 subset ( $p < 0.05$ ; Figure 5.11). Again the area graph in Figure 5.11bii showed the same trend but the data was not significantly different. The cells with YM022 added first at day 4, did not show a significant difference in sphere number compared to the untreated cells.

In summary, the knock-down of CCK-2R did not affect sphere-forming in colorectal cancer cells. However, inhibiting CCK-2R before HOP-62 or U251 cells formed spheres did cause this development to be delayed until days 4 and 6, respectively. Adding YM022 after the first spheres had formed (at day 4) did not have any effect on colorectal or lung cells, nor did it have the same consequence as adding YM022 to HOP-62 cells at day 1. Therefore, in the lung cell-line, YM022 delayed sphere-forming but may have also affected sphere maintenance a small amount since adding YM022 at day 4 was statistically similar to both the YM022 and untreated groups. However, in U251 cells adding YM022 at day 4 was significantly different to adding it at day 1, but not compared to no treatment. Hence in the glioma cell-line, YM022 delayed sphere-forming but did not affect sphere maintenance at all.

### 5.10. Summary

- All of the cell-lines developed into 3D sphere structures when plated in SCM; however these structures differed with cell-line and cancer type.

- CCK-2R gene expression was up-regulated by 3D culture of lung and glioma cell-lines and time was a significant factor in the difference between monolayer and sphere expression levels in colorectal and glioma cell-lines.
- CCK-2R protein was also up-regulated in spheres at day 10, and seemed to be located in the outer cells of each sphere.
- Gastrin, CD133,  $\beta$ -catenin and CD44 expression did not seem to be correlated to that of CCK-2R.
- $\beta$ -catenin protein did not seem to be localised to the nucleus of the sphere-forming cells.
- Growing cells in 3D culture did not have an effect on CCK-2R promoter activity.
- In 3D culture, inhibition of CCK-2R using YM022 successfully delayed sphere-formation in HOP-62 and U251 cells, indicating a role for the CCK-2R in glioma and lung cancer stem cell expansion.



## Chapter 6 Discussion and conclusions

### **6.1. LATE-PCR is a sensitive method for detecting the CCK-2R at the gene level**

The CCK-2R is up-regulated in cancer and mediates many carcinogenic processes, allowing a cancer to progress rapidly. This potentially makes it an important biomarker in cancer, and therefore an understanding of the expression patterns, effects and regulation of the CCK-2R are important to improve our understanding of its role in this disease.

The CCK-2R acts as the receptor for gastrin, a hormone shown to prevent apoptosis and increase angiogenesis, cell proliferation and metastasis in cancer cells [81]. Gastrin is also up-regulated in cancer cells resulting in a high degree of activation of these multiple pro-carcinogenic pathways. Using specific CCK-2R inhibitors, it has been proven that carcinogenic effects can be lessened when the receptor is blocked [13, 74, 138, 139].

CCK-2R is therefore detectable at the protein level in carcinoma cells but detecting its gene expression in cell-lines, and in patient tumour samples, is a challenge. Many studies have used classical PCR to detect the CCK-2R within cancer cells; however, at times important negative controls were not included [24], which are crucial in order to rule out any false positive outcomes. This is due to the small size of the introns within the CCK-2R gene, which allow easy amplification of CCK-2R genomic DNA which frequently contaminates RNA preparations [179], within the timescales of qRT-PCR. The Taqman qRT-PCR, initially used to detect endogenous transcription of the receptor, could only do so in the minority of cancer cell-lines. In the panel of cell-lines tested in the present study, H209 cells (SCLC) expressed the highest level of endogenous CCK-2R; a result supported by the literature. Moody & Jensen [180] found that both the endocrine-mediated proliferation of SCLC caused by CCK8 and SCLC basal growth

(autocrine stimulation by gastrin) were inhibited by the CCK-2R inhibitor, CI-988 (PD134308). Another study of lung cancer cells showed that the CCK-2R was present in 6/7 SCLC (although this panel does not include H209), and not at all in non-SCLC (NSCLC), using classical PCR [159].

Using the Taqman assay OE33 cells in the current study showed low expression of the receptor, whereas OE19 cells did not appear to express it at all. However, Harris *et al.* have shown that both these cell-lines possess the CCK-2R by IHC staining and functional assays [13]. This indicated that perhaps the 'receptor-negative' cells in the panel show low expression and that the Taqman assay was not sensitive enough to detect these.

A novel method, LATE-PCR, which is based on asymmetric PCR, was established in the current thesis. LATE-PCR was originally developed by a group headed by Wangh [162], in order to detect small mutations in genes which convey two serious human conditions; Tay-Sachs disease and cystic fibrosis [160]. Both of these disorders are caused by a 3 or 4 base pair change in a single gene, which is detectable from the single cell of each embryo, tested via a sensitive assay such as LATE-PCR [162]. As with the CCK-2R, there is a very small amount of the molecule of interest (CCK-2R mRNA or embryo DNA) present, and yet the protein produced is of critical importance to the cell.

LATE-PCR was found to be 10,000-fold more sensitive at detecting the CCK-2R than the Taqman assay. Using LATE-PCR all of the human cell-lines in the panel were shown to be CCK-2R-positive, as opposed to the 5/7 shown as positive with the Taqman assay. The cell-line panel was extended to include other cancer types such as breast carcinoma and glioblastoma, but H209 cells still had the highest endogenous

expression; at least 10-fold higher than the other cell-lines. The 3T3 wt cell-line was used as a negative control, because it is derived from mouse fibroblast cells and therefore should not contain human CCK-2R mRNA. The LATE-PCR assay showed this cell-line as undetectable as it had in the Taqman assay providing support for the specificity of this assay.

When dilutions of CCK-2R mRNA were tested by the LATE-PCR assay, it produced a linear regression line with a slope of 1.03, showing a ten-fold change in signal for a ten-fold change in dilution. In the entire panel of 35 human cancer cell-lines, two were found to have no expression of CCK-2R; ST16 (gastric) and BT4 (paediatric glioma). In the cell-lines with CCK-2R expression the extremes were shown by H209 cells with high relative expression levels ( $2^{-\Delta Ct}$ ) of  $10^{-2}$ , and AGS (gastric) cells with  $2^{-\Delta Ct}$  at  $10^{-6}$ . The other colorectal, gastric, pancreatic, oesophageal, glioma, lung, ovarian, melanoma and breast cell-lines had relative expression levels of between  $10^{-3}$  and  $10^{-5}$ .

The gastric cell-lines had extremely low expression of CCK-2R, which was unexpected due to the large role this receptor plays in normal stomach tissue [135]. This trend was taken to the extreme by ST16 cells, where no receptor was detected; showing that either no CCK-2R mRNA is present or that the assay cut-off point is too high to detect its very low level expression. In this project A549, EKVX, H23 and HOP-62; all NSCLC cells, have been found to express CCK-2R, whereas no NSCLC cell-lines had been shown to express CCK-2R before [159]. This shows the sensitivity of LATE-PCR in detecting the CCK-2R where it has not been found previously.

Matheiu & colleagues [181] state that no human glioblastoma cell-lines have been found to express the CCK-2R, and yet gastrin can modulate

apoptosis, proliferation and motility of these cells *in vitro* [31]. It is possible that this effect is not mediated via the CCK-2R, of course, since other gastrin receptors, including CCK-2R isoforms, have been described [182]; however, this seems unlikely since the brain is one of only two sites where the CCK-2R is highly-expressed in non-malignant tissue [135]. Consequently, including the human glioblastoma cell-lines was a high priority since the new assay might be able to give insight into how gastrin mediates its effects in this cell-type. As with the NSCLC, these cells were found to express CCK-2R mRNA, with U251 and U373 cells at the upper and lower ends, respectively, of the normal range found in the cell-line panel ( $2^{-\Delta Ct}$  between  $10^{-3}$  and  $10^{-5}$ ). This demonstrates that the CCK-2R is present in gliomas and may mediate the effects of gastrin in this tumour-type.

Another interesting result is shown in the paediatric glioma cell-line, BT4, where no expression of CCK-2R was found. This cell-line also yielded no gene expression of CD133, which was unexpected due to the apparent necessity of CD133<sup>+</sup> cancer stem cells in the formation of adult gliomas [46]. The other paediatric glioma cell-line in the panel was OLIG1 [45], which showed CCK-2R gene expression in only one passage (of two), and no CD133 expression in either passage (results not shown). This indicates that perhaps both CCK-2R and CD133 are different in the brains of children and adults, and that our primers are not able to detect them reliably in the paediatric situation. A study by Ma *et al.* showed that CD133 mRNA expression could be detected by real-time RT-PCR in primary astrocytoma samples, some (14/72) of which were paediatric [183]. We assume that CD133 expression was found in these paediatric samples, since they do not say otherwise and also that CD133 expression is similar in astrocytomas to glioblastomas. In the current project both CCK-2R and CD133 protein have been shown to be present in these cell-lines.

The fact that the CCK-2R could have a different conformation in BT4 cells broaches the possibility that a different receptor isoform could be encoded in these cells. Detection methods, such as radiolabelled peptide targeting, for the well-known CCK-2Ri4sv have been validated in colorectal cancer [101, 105] and LATE-PCR could be adapted to target the retained intron 4 (being aware of the potential for gDNA amplification). However, this and the other isoforms described in the literature [121-123] would be detected by the LATE-PCR described in Chapter 3, and so should not be present in BT4 cells. Perhaps there is yet another isoform, or maybe paediatric brain tumours take low expression of CCK-2R mRNA to an extreme below even the level of detection of the present LATE-PCR assay.

A further way to investigate why expression of CCK-2R is different within cancer groups would be to look at a correlation with their mutational status. The CCK-2R mediates the link between gastrin and the Wnt pathway [184, 185], where many oncogenes are found e.g.  $\beta$ -catenin. There may be a relationship between the expression of the CCK-2R and certain oncogenes, which could not be investigated without this highly sensitive method for detecting CCK-2R mRNA. If this were the case it would confirm the need for a therapeutic approach to CCK-2R knock-down.

In order to validate the LATE-PCR assay, siRNA molecules targeting the CCK-2R were designed and optimised to knock-down the receptor. These siRNAs were very successful, reducing CCK-2R expression to between 3 and 30% of the control in gastric, colorectal, glioma and lung cancer cell-lines. This reduced expression could be detected by LATE-PCR whereas the Taqman assay failed to perceive the signal.

The siRNA molecules were also created with the aim of investigating the biological effect of CCK-2R knock-down on cancer cells. The ligand for CCK-2R, gastrin, was investigated at the gene level since these two molecules are linked in an autocrine loop [137, 186]. No difference was found in gastrin gene expression between cells displaying CCK-2R knock-down for 24 hours and the negative control siRNA-transfected cells. CD133 gene expression was also investigated to test the hypothesis that CCK-2R is involved in cancer stem cell growth or maintenance. However, again at 24 hours, there was no change with the decrease in CCK-2R gene expression. These results may have been due to the length of the experiment, since CCK-2R protein levels may not be affected within 24 hours. It seems the genes investigated are not linked to CCK-2R at the transcriptional level, but they may be translationally linked. If the CCK-2R mRNA needs to be translated to protein to affect either gene or protein levels of gastrin/CD133 this may need more than 24 hours.

During optimisation of the flow cytometry protocol used in this project, CCK-2R protein expression was investigated following receptor siRNA-transfection. This initial experiment failed to show a change in cell-surface protein expression (by live cell staining) up to seven days after knock-down, and due to time constraints this was not explored further once an effective flow cytometry procedure was in use. This problem highlights the need for a more reliable method of knock-down than the transient transfection, which may not reduce CCK-2R mRNA levels sufficiently that translation is stopped entirely. Since translation of CCK-2R is likely to be very efficient (see Section 6.2 below), a superior method for removal of CCK-2R transcription, such as a viral inducible knock-out construct, should be investigated. This would allow the cells to recover from transfection before further studies were carried out, and enable analysis over a longer time period to be investigated. An example of this system is the inducible knock-out of PTEN in breast cancer cells, where total knock-out caused growth suppression and cell

death [187]. The inducible construct provided a way around this problem, allowing PTEN to be turned off at a point where the mechanism of growth suppression could be monitored and investigated [187, 188].

LATE-PCR has allowed the design and use of CCK-2R siRNA in these cancer cell-lines. With advances in siRNA technology taking place, it is becoming more likely these molecules could be used as therapeutic agents [189] and it is possible that knock-down of the CCK-2R in a clinical setting could be an appropriate treatment for specific forms of cancer. Combined with the specificity of LATE-PCR, it may be possible to determine whether a certain individual would respond to this treatment, therefore allowing a personalised therapeutic recipe to be designed.

## **6.2. CCK-2R protein expression is not correlated to gene expression**

CCK-2R protein expression was investigated in a smaller panel of cell-lines, comprising colorectal, glioma and lung cancers. These cell-lines were chosen due to the fact that each of these cancer groups encompassed cells with high, low and average CCK-2R gene expression; in order to give an insight into whether gene expression was linked to translation. Protein expression was visualised and quantified using live cell staining with an antibody to an external loop of CCK-2R, followed by flow cytometry. Two populations of cells were found within each cell-line; one weakly-positive and the other strongly-positive, possibly indicating diverse differentiation among the cells. A similar situation was shown in various cell-lines, where strong expression of CD133 has been associated with resistance to apoptosis and the CSC subset [190]. This hypothesis is investigated further in Section 6.4 below.



Two values were obtained from the flow cytometric analysis; the median fluorescence for the whole population, and the percentage of highly-positive cells in the population. The percentage value was more reproducible between replicates and therefore showed tighter error bars than the medians. It was also cancer-type specific, with colorectal cells showing a population of ~20% highly-positive cells and glioma and lung cells showing around 40%. The staining was verified by carrying out negative controls with only secondary antibody added, and by undertaking a primary antibody dilution experiment. The negative control samples always had far lower FITC fluorescence and dilution of the primary antibody reduced fluorescence clearly showing that the antibody binding was specific for the CCK-2R.

The protein expression showed a three-fold range compared to the four-log range in gene expression, leading to a linear regression line which was almost horizontal since gene expression varied and protein expression was fairly similar across the panel. Therefore CCK-2R gene expression could not be correlated with protein expression. Since protein expression is easily detectable it is not clear why the level of gene expression is so low in the cell-line panel. It was hypothesised that the LATE-PCR results could demonstrate one of three different scenarios; a low level of receptor mRNA in all cells, a large amount of mRNA in just a few cells or a mixed population of cells; most with a very low level of mRNA and a few that have a large amount present. Since the flow cytometry results in this thesis show the whole population to express the receptor protein, it would appear that the CCK-2R mRNA is present in all the cells, but perhaps it is not distributed equally. This could be investigated if the protocol to transfect the LATE-PCR molecular probes into the cancer cells could be optimised to show where and how much mRNA is in each cell.

The fact that protein is being made from low levels of mRNA in most or all of the cell population suggests that the gene is being translated very efficiently. Many oncogenes are up-regulated due to a tumourigenic increase in the mRNA cap-binding protein, eIF-4E, which allows translation to happen more swiftly [191]. This has been demonstrated on anti-apoptotic proteins in lung [192] and growth promoting proteins in colon cancer [193] along with many others [191]. It is possible that eIF-4E may be responsible for the efficient translation of the CCK-2R, and this could be explored using qRT-PCR.

Korner *et al.* also found a non-linear relationship between CCK-2R gene and protein expression, assessed using qRT-PCR and receptor binding site autoradiography, respectively [194]. They suggest that these findings may be due to translational regulation or collecting the receptor protein into intracellular pools [194, 195]. However, the latter explanation would not tie in with the current project as it indicates that the protein would not be on the cell surface and therefore could not be detected by live cell staining. Perhaps receptor protein levels and amount of receptor binding sites in colorectal tumours are also not correlated. Although unlikely in normal cells where translation is tightly controlled, this is a possibility in cancer cells since regulatory pathways are often damaged.

Another hypothesis is that the CCK-2R mRNA is very unstable, therefore making detection difficult. mRNA stability is usually tightly controlled by the communication between *cis*-acting elements and *trans*-acting factors [196]. mRNA molecules can be long-lived or they may be degraded directly following transcription, which can happen in the immune response [197]. Here the expression of cytokines and growth factors must be tightly controlled, with translation being turned on and off rapidly to enable a swift response with no autoimmune symptoms. This is achieved through transcriptional and post-

transcriptional regulation. However in carcinogenesis, post-transcriptional regulation can be disrupted, for example an AU-rich element (ARE) binding protein, HuR, is up-regulated in colon cancer, allowing the stabilisation of COX-2, and consequently an increase in angiogenesis and invasion of neighbouring tissues by the tumour cells [198].

This hypothesis may also explain why promoter activity was not related to gene or protein expression in this project. If the CCK-2R gene is being transcribed quickly but the transcript is being rapidly degraded then the fact that the promoter is active would bear no relation to the amount of mRNA present. The most apparent case was that of BT4 cells where promoter activity was 30 times that of the control, despite a lack of any gene expression detected by LATE-PCR. However, in real-time PCR assays, mRNA transcribed by the cell is measured at a certain time-point. These assays are limited in that they cannot demonstrate what happens to temporal mRNA expression.

Therefore RNA stability may play a major role in the regulation of the CCK-2R, and this could be investigated in further work. Cheneval & colleagues review several methods for studying mRNA stability, including the use of a branched DNA assay to directly measure mRNA at different time-points and a chimeric gene sequence to show transcript abundance after inhibition of transcription, compared to a house-keeping gene [196]. Real-time PCR is a sensitive and reliable method to find out the half-life of mRNA molecules with low expression, measuring these relative to total RNA [199].

Translational control may also be important to consider since even small amounts of mRNA may be translated to yield significant amounts of protein, and such mechanisms may come into play in stress

conditions. For example, it has been found that in hypoxia, translation of most mRNAs is down-regulated, but that of activating transcription factor 4 (ATF4) is enhanced as a survival mechanism [200]. ATF4 then activates genes involved in the stress response, such as those required for DNA damage repair. In order to investigate the possibility that stress conditions change CCK-2R promoter activity, the constructs were tested in hypoxia and serum-starvation. As before, with the gene expression studies, no difference in promoter activity was found in either of these conditions, after 24 hours. This could be investigated further, using longer time-frames, together with analysis of stress proteins to confirm the level to which the cells are stressed.

Once gene and protein expression levels of CCK-2R had been established, it was decided to investigate the location of CCK-2R transcripts in the cell. mRNA should be strongly associated with ribosomes in order to ensure efficient translation, but it has also been suggested that molecules with low mRNA and high protein may have their mRNA stored in the nucleus [201]. In order to try to localise the CCK-2R mRNA inside the cell, a method was devised to transfect the fluorescent LATE-PCR probes directly into the cells. These probes would then fluoresce and identify the location of the mRNA under a microscope. Unfortunately, although the method showed initial promise, it was abandoned due to lack of effectiveness with housekeeping genes HPRT and GAPDH. The protocol needs further optimisation, focusing on the transfection reagent and possibly modification of the probes to encourage transfection. A review of the current methods used to increase transfection efficiency is provided by He *et al.* [202], for example adding poly(ethylene-imine), PEI, to the probes, to enhance cell uptake. Another possibility would be to add a lipid molecule to the probe, forming a complex in a more acceptable shape, again improving uptake across the cell membrane [202].

LATE-PCR has demonstrated that CCK-2R can be detected at the mRNA level in cancer cell-lines, where previous studies had only found the receptor protein, but it was important to test this assay in primary tissues. It is possible that expression is artificially low *in vitro*, as opposed to *in vivo* where environmental effects could cause up-regulation. Colorectal primary tumours, liver metastases and lung primary tumours were tested along with their equivalent normal tissue, and all the groups were found to have some samples with expression of CCK-2R mRNA. This was extremely low in normal lung tissue, however, and the lung primary tumour samples showed significantly increased (100-fold) CCK-2R gene expression compared to the normal tissue. It was also the opposite of the CCK-2R expression in the cell-line panel, where colorectal cells show higher expression than lung cell-lines (except the SCLC line, H209). This may be an indicator that the cell-lines do not truly represent the *in vivo* situation.

Korner *et al.* corroborate these results, in that they found CCK-2R receptor mRNA in colorectal, liver and lung tumour tissue (again slightly higher in lung than colorectal tumours) as well as in colorectal and liver normal tissue samples [194]. They did not find CCK-2R at all in the lung normal mucosa. Their lung tumour samples were a mixture of SCLC and NSCLC, whereas those examined in this study were NSCLC subtypes including adenocarcinoma and squamous cell carcinomas. Other groups have found high expression of CCK-2R in lung cancers, (SCLC) but they have not investigated the expression in equivalent normal tissue [203, 204]. Clearly further work must be carried out to discover whether CCK-2R could be a prognostic marker in lung cancer, and if this knowledge could be used therapeutically.

The evaluation of a putative prognostic marker is a complicated process. In this case it could be carried out either retrospectively; with samples already collected and stored, or prospectively; by enrolling

patients as they are diagnosed with lung cancer [205]. The second is a superior method, since it allows the collection of relevant patient data and uses unbiased selection; however, it would take much longer, since lung cancer is a disease with a long duration. Other factors to consider are the ethics of the study and sufficient sample size to provide an accurate and adequate answer to the hypothesis in question [205].

Gastrin gene expression was also investigated in these primary tumours and although there were no significant differences between tumour and normal tissue, there were some correlations with CCK-2R expression. The primary colorectal tumour showed a link between expression of these two genes, whereas normal colorectal (and all the other sample groups) did not. Gastrin and the CCK-2R are linked in an autocrine loop in gastric carcinoma cells [137], and these results suggest that there is a connection between expression of these molecules in colon cancer cells. The fact that they are not linked in colorectal normal tissue, but are in colorectal carcinoma tissue implies that the relationship is promoted by tumourigenesis. Zhou *et al.* blocked the autocrine loop in gastric cells, by knocking down the CCK-2R and by counteracting the secreted gastrin they found in the culture medium, thus reducing cell proliferation [137]. This could be considered as a further study to continue the work of the current project in colorectal carcinoma cells.

### **6.3. The expression of CCK-2R is regulated via its proximal promoter and up-regulated by drug resistance**

The regulation of the CCK-2R is not well-researched; however, this topic does seem to be gaining interest. Since we began this project, Ashurst *et al.* [114] published a study using CCK-2R reporter constructs. They used these constructs to determine those sections of

the putative CCK-2R promoter which could maintain transcription, and to establish the importance of the consensus binding sites for several transcription factors found within it (see Section 1.6). They also found that environmental factors such as serum-starvation can have an effect on the promoter.

The constructs designed in this project have shown that the 500bp proximal to the start codon for the CCK-2R gene are active when transiently transfected into colorectal, glioma and lung cancer cell-lines. The constructs containing 500 or 250bp before the start codon for CCK-2R had most activity in all the cell-lines. The 1kb construct, CCK2R-1057, was also found to have promoter activity in the colorectal and A549 cells, but this was always much less than that of the smaller constructs. The 1kb deletion construct, which contains the same fragment as CCK2R-1057 minus the 260bp closest to the start codon, has no activity at all, corroborating the major role for this small section of proximal promoter.

This research could be explored further using computer programmes such as ALGGEN PROMO [206], to predict transcription factor binding sites. This information could then be used to decipher which parts of the 500bp are most critical to promotion of the CCK-2R gene. A further way to investigate the transcriptional regulation of the CCK-2R would be to incorporate GFP into the promoter constructs; to develop a dual reporter in order to study the location of promoter activity within the cell population, in real time [207].

Within the tumour environment, conditions such as oxygen concentration [208] and nutrient availability [209] are reduced, due to decreased vasculature [167]. In order to simulate these stress conditions, colorectal and glioma cell-lines were grown in hypoxia (1%

oxygen) or serum-free medium, and their CCK-2R gene expression was compared to that of cells growing in normal conditions. HCT116 cells showed a trend of two-fold CCK-2R gene up-regulation in both hypoxia and serum-starvation, but there were no significant differences in expression in any of the cell-lines. Transcription of the CCK-2R gene does not seem to be affected by 24 hours of stress, and these results were corroborated when promoter activity also showed no significant differences between normal conditions and either hypoxia or serum-starvation.

Hypoxia was verified by assaying for the HIF-1 $\alpha$  protein and also VEGF gene expression, an indicator of HIF-1 $\alpha$  induction, but serum-starvation was assumed. These experiments were carried out for 24 hours, which may not have been sufficient time to see a change in expression levels. This time-point was chosen since it was enough time for Ashurst & colleagues to see an increase in CCK-2R expression in serum-starvation [114]. However, that study was carried out in a rat pancreatic cell-line, AR42J, with a Taqman qRT-PCR assay for rat CCK-2R, which may not mimic exactly the human condition. If this experiment were repeated the cells should be stressed for longer times and stress proteins, such as AGR2 should be assayed for, in order to ascertain the level of stress in the cells [209]. Another possibility with this experiment is to use protein expression as the reporter for CCK-2R up-regulation, since this showed a more significant difference in the 3D studies (see Section 6.4 below), and may be affected independently of gene expression. A reporter of translation, encoding GFP inside 5' and 3' untranslated regions of the CCK-2R, could also be used for this purpose.

Drug resistance is another characteristic of cancer cells, particularly CSC, and it was hypothesised that CCK-2R may be up-regulated in cells developing resistance to drugs. The chosen drug was cisplatin; a



chemotherapeutic used to treat many carcinomas, and one where drug-resistance is very common [210]. HCT116 cells were exposed to increasing concentrations of cisplatin, and considered cisplatin-resistant when grown in 1µg/ml cisplatin for 3 passages. The cisplatin IC<sub>50</sub> of these cells was compared to that of the wild-type HCT116 cells, and found to be higher in each experiment although this difference did not reach significance. When LATE-PCR was used to detect CCK-2R gene expression in these cells, it was found to be almost three-fold higher than in the wild-type cells ( $p < 0.05$ ). However, knock-down of CCK-2R did not have an effect on cisplatin sensitivity, showing that the receptor is up-regulated in drug resistance but not fundamental to the preservation of this characteristic. This may indicate that the CCK-2R is a surrogate marker for drug resistance.

Surrogate markers for drug-resistance are very important in cancer research since they can allow the prediction of drug-resistance before the event. GRP78 over-expression was found to be linked to resistance to adriamycin in breast cancer, consequently if a patient displayed this characteristic then alternative therapies were considered [211]. A new surrogate marker has recently emerged during studies of ovarian carcinoma; <sup>18</sup>F-FLT PET uptake correlates with mTOR inhibition in cisplatin-resistant tumours [212]. Further studies with the CCK-2R could be carried out, using qRT-PCR to determine the gene expression profiles of known drug-resistant primary tumour samples to see whether a correlation exists.

#### **6.4. Expression of the CCK-2R is up-regulated by, and involved in, growth of cells as tumour spheres**

Cancer cell-lines are classically grown in 2D monolayer culture; however, the literature is beginning to show that this method is not representative of the *in vivo* condition. In the human body solid tumours

are 3D structures comprised of many different cell types and environments, often including a hypoxic centre. If tumour cells could be grown in 3D, then they could more truly represent the natural situation [173, 174]. Lee *et al.* have demonstrated the similarity of 3D models and real tumours, in terms of gene profiling [44], compared to 2D models in primary glioma tissue. The genes profiled included CSC markers such as CD133 and Sox2, proliferation markers and CNS development markers. The spheres were formed from primary tumour samples, unlike those in our study, and they suggest that transformed cell-lines lose their CSC subset over many passages [44]. However, they do reference several studies, e.g. [213], where CSC have been found within tumour cell-lines, and so suggest that these cells may be encouraged to grow back from a very small population or indeed re-emerge from differentiated cells in the correct conditions for their growth.

There are several methods in the literature to achieve the goal of growing tumour cell-lines as 3D structures. The method used in our study, and that described above, involved using a serum-free medium, supplemented with EGF and FGF (SCM), to encourage sphere growth and to select for stem-like cells. In the literature the first neurospheres were formed in this way; Reynolds and Weiss discovered that neural cells grown with EGF formed clusters of cells, and that these cells differentiated into more than one cell-type, proving their stem cell capability [176]. This method uses serum-free conditions to limit the normal differentiation of cells in serum-containing medium, and Lee *et al.* also found that telomerase activity was maintained in this special medium [44]. Telomerase is an enzyme which adds sections of DNA to the end of chromosomes which are otherwise lost during replication, allowing a cell to continue replicating far longer than those without telomerase. This activity and the limitation of differentiation are stem cell characteristics, and therefore in tumour cell-lines the SCM method is enriching for cancer stem cells. Another tumourigenic, and possibly

CSC, characteristic is the ability to invade nearby tissues and the xenografts made by Lee *et al.* from sphere-forming cells had far more capacity for metastasis than did xenografts of cells grown in 2D [44].

Neural cells are not the only cell type to display similarities to the *in vivo* situation when grown as spheres. Tumour cells from the colon which were maintained in 3D culture using the SCM method produced tumours with the same pattern of  $\beta$ -catenin and cytokeratin 20 (CK20; a differentiation marker) expression, and histological features, when transplanted into mice [47]. Equivalent cells grown in 2D culture conditions did not show these characteristics and the CD133<sup>+</sup> subset was selected against, unlike in 3D culture [47]. Lowthers *et al.* grew HT29 and two other colorectal cell-lines as tumour spheres in SCM, and found that these cells were far more resistant to the drug ceramide than those grown as monolayers [214]. Interestingly when they investigated whether this was due to lack of perfusion through the densely-packed cells in the sphere they concluded it was not, because 5FU and cisplatin were found to be equally effective in both their 2D and 3D cultures [214]. Therefore it would seem that growing cells as spheres does not induce resistance to all drugs.

An alternative method for 3D culture is to provide a support basement membrane matrix for the cells to grow around, forming spheroids. The terms sphere [44, 47, 173, 176] and spheroid [174, 175] are both used in the literature to describe a 3D cellular structure *in vitro*; however, in this project the two terms will distinguish between those formed using a serum-free medium or a support matrix, respectively. Smalley *et al.* describe that they have seen large differences in mRNA profiles between 2D and 3D cell cultures [174], and that fibroblasts grown on a matrix show a morphology much like that *in vivo*, however the rigidity of the matrix can cause cell organisation and signalling to change. This is

a weakness of the method since the characteristics which can be altered by the matrix are those being measured.

Two other sphere-forming methods were examined by Liu *et al.* where cells were encouraged to aggregate by the application of ultrasound waves [215] or gyratory force [216] to the culture medium[175]. The ultrasound waves caused the medium to vibrate pushing the cells towards the middle of the well, and allowing the aggregates thus formed to be grown in normal growth medium [215]. The gyratory method also allows this with the cells being grown in rotating vessels [216]. A549 cells grown in this 3D culture mimicked primary tumour immune responses; they had increased lipopolysaccharide receptors for detecting bacterial molecules [175]. However, the gyratory method was first developed to form aggregates of liver cells in order to investigate the processes carried out by 3D multi-cell-type models compared to the original organ [216]. This is a very different goal to enriching for cancer stem cells in tumour cell populations, since existing cells are forced to aggregate rather than growing a sphere from one, or very few, cells. Both these models would be useful if one were to investigate the interactions of different cell-types within a tumour population, but are of little use when studying the cells which initiate a tumour, as in this project.

In the current study sphere cultures made from colorectal, glioma and lung cell-lines were investigated, since these cell-lines showed differing amounts of endogenous CCK-2R gene expression. If CCK-2R expression was linked to the CSC phenotype then higher levels of CCK-2R endogenous expression may relate to larger spheres or an increase in CCK-2R expression in those spheres. CCK-2R gene expression was found to be increased in day 10 spheres in U373, A549 and HOP-62 cells. These spheres adhered to the plate almost up to day 10 of

growth, were perfectly round and showed filaments while they were attached to the plate.

In HCT116 (wild-type and cisplatin-resistant), HT29 and U251 cells there was an increase in CCK-2R expression by day 10 but this was mirrored in the monolayer cultures, possibly due to the fact that these had reached 90% confluency by that time. The colospheres grown from HCT116 and HT29 cells were rounded, had a well-defined outer edge and floated in the medium soon after formation. They were much larger than those formed from lung or glioma cell-lines, indicating that morphology, i.e. a smaller, more adherent sphere, may be related to CCK-2R up-regulation. This is also interesting since Vermeulen *et al.* hypothesise that HCT116 cells do not contain a CSC subset and therefore should not produce spheres [53]. U251 spheres looked different from the colospheres, and more similar to those of BT4; irregularly shaped but with a defined edge, but CCK-2R gene expression could not be detected in BT4 cells at any time or culture. Possible reasons for the lack of expression are discussed in Section 6.1 above.

CCK-2R protein expression was significantly up-regulated in spheres formed from HCT116, HT29, BT4 and U373 compared to 2D culture, and the trend was also shown in U251 cells, although this was not significant. These results were shown by the median FITC fluorescence by membrane-staining, and the values for percentage of CCK-2R<sup>hi</sup> cells show the same trend but not the same significance. This would indicate that the cells in the sphere cultures express CCK-2R more strongly, rather than there being significantly more cells expressing CCK-2R overall, than in the 2D culture.

Paraffin-staining showed that CCK-2R expression is highest in smaller spheres, and that it is mostly restricted to the cells at the edge of the sphere. Yeung *et al.* investigated CSC and differentiation markers in their colorectal spheroids and found that Ki67 was expressed at the outer edges of the structures [217]. This was expected as it shows that the rapidly dividing cells are at the outer edges, with the CSCs at the centre; if this were the case, it would mean that the cells which highly express CCK-2R are not the CSC subset.

The centre of a sphere/spheroid is more hypoxic than the edge and many studies have shown hypoxia to select for CSCs [218]. Acker & colleagues reported that the  $pO_2$  of the centre of a sphere was much lower than that of the outer cells, especially of colospheres such as those formed by HT29 cells [219]. The current finding that CCK-2R is not found at the centre of the sphere corroborates the result in Chapter 5, where CCK-2R was not found to be significantly up-regulated in hypoxia. The fact that the larger spheres, those formed by colorectal cells; assumed to have more hypoxic centres, show less up-regulation of CCK-2R gene expression than the smaller lung and glioma ones also points to this conclusion.

A further result to confirm the finding that CCK-2R is not expressed in the CSC subset is that CCK-2R expression is not correlated to that of CD133, CD44 or nuclear  $\beta$ -catenin in the spheres. CD133 protein was up-regulated six-fold in BT4 spheres, did not change in U251 or HOP-62 spheres and was actually down-regulated in the colospheres. CD133, CD44 and nuclear  $\beta$ -catenin are accepted CSC markers, at least for glioma and lung cancer, and are highly implicated in colon carcinoma too [43, 46-48, 57, 58]. Unexpectedly, CCK-2R and gastrin gene expression are not related either, despite the autocrine loop which links them in gastric cancers and glioma (U87 cells) [137, 186]. This non-correlation had been found earlier in monolayer cells, so the

autocrine loop may be present at the protein level only or may require gastrin stimulation in the cell-lines used in this project. Growing cells in 3D had no effect on receptor promoter activity despite the up-regulation of CCK-2R transcription in these conditions.

Many groups have used the SCM method to investigate CSC within several different types of tumours, although they have not looked at CCK-2R expression. Hussein *et al.* reported that neurospheres formed from paediatric glioma, ependymoma and other brain tumours contained a stem cell subset with Sox-2 and membrane CD133 expression [45]. This study was carried out in the same way as ours, by re-suspending monolayer cells in SCM. Immunofluorescence (IF) showed them that although CD133 protein overall had not been up-regulated, membranous CD133 had been increased four-fold [45]. This corroborates with the present project where paediatric BT4 spheres showed up-regulation of CD133 protein, but does not match with the adult U251 sphere result. Qiang *et al.* found that CD133 protein increased from 4 to 6% of cells at day 10 of sphere growth in U251 cells treated the same way, but they do not state that this difference was significant [220]. This may indicate that within glioma cells at least, paediatric cells have a higher percentage of CSC present, and are therefore more tumourigenic than their adult counterparts.

In a study described above, Ricci-Vitiani *et al.* found that all the cells within their colospheres maintained CD133 expression and lacked CK20, demonstrating that they were not differentiating [47]. However, in another study, again with spheres grown direct from primary colon tumour tissue in SCM, the cells within the spheres were found to have different phenotypes; a small number of stem cells were maintained with many differentiated cells making up the rest of the sphere [221]. This is a more likely occurrence since CSC divide into one stem cell and one daughter cell; it should be impossible to maintain a CSC-only

population. This heterogeneous population theory is borne out by the finding in our sphere cultures that CCK-2R protein expression is higher in certain cells rather than expressed in more cells within the spheres. However, it appears from the location of those cells that they are not the CSC subset.

Vermeulen *et al.* showed very low levels of nuclear  $\beta$ -catenin in their spheres by IF [221], which may shed light on our finding that  $\beta$ -catenin is cytoplasmic in the tumour spheres. Unfortunately they do not suggest a reason for the lack of stemness in some of their sphere cultures, other than that the CSC niche may play a role in deciding when the cells differentiate and lose their CSC characteristics. This cannot be applied to our project since the CSC niche would not be present in *in vitro* culture. The IHC staining for  $\beta$ -catenin carried out in this project has not been verified, although positive control (pancreatic carcinoma) tissue was used for optimisation. Also the method for embedding the spheres in paraffin may need further modification to ensure the preservation of the spheres and therefore the accuracy of the staining.  $\beta$ -catenin expression could be investigated further using the IF protocol described by Vermeulen *et al.* [221].

Our data on colorectal monolayer cells expressing higher levels of membranous CD133 protein than those grown as spheres is at odds with the established literature [47, 221]. This could be due to the antibody not recognising the correct epitope on the CD133 protein, however, this is unlikely as it was taken into consideration. The antibody used for the staining recognised the AC133 epitope of the CD133 molecule [222], which has been proved to be vital when distinguishing CSC, since this is lost in differentiated cells [223]. Another possible explanation is that the colospheres were grown from cells which were at too high a passage and therefore terminally differentiated, but if this was the case they should not have grown into spheres.



Lung tumour spheres formed from lung cancer cell-lines in SCM have been found to contain cells more resistant to cisplatin than cells grown as a monolayer and to exhibit nuclear  $\beta$ -catenin expression [224]. These lung sphere cells were enriched for CD133 and Oct4, and could produce xenograft tumours with significantly less cells than those grown in 2D culture [224]. This draws attention to the fact that there are many other CSC markers which have been suggested to imply a CSC phenotype; not just Oct4 but Nestin, Sox-2, Lgr5, ALDH1, CD166 and ABCG2 [43, 52, 54, 55], which could be used to further investigate the CSC subset within the tumour spheres in this project. By far the best method to conclusively test whether the CCK-2R<sup>hi</sup> cells are the CSC subset would be to isolate them and grow xenograft tumours in immunodeficient mice. If the CCK-2R<sup>hi</sup> cells can produce tumours from 100-1000 cells then this would place them in the tumour-initiating-cell category and therefore they would be CSC [52].

In order to investigate whether lack of CCK-2R signalling had any effect on sphere-formation or maintenance, an experiment was carried out using an inhibitor of CCK-2R signalling, YM022, a highly selective antagonist for the CCK-2R [178]. Initially siRNA-transfected cells (both CCK-2R siRNA and the control negative siRNA) were used but they grew into spheres at such a slow rate that no effect of CCK-2R knock-down could be observed. Using siRNA knock-down would have raised concerns because transient transfection may not have kept CCK-2R expression low enough for the time-frames involved in sphere-forming (ten days in this project). If the gene had returned to full transcription before the end of the experiment the results would have been skewed. Also, as discussed above, CCK-2R may demonstrate very efficient translation which may not have been ceased completely, whereas a protein inhibitor should stop the signalling pathway immediately and can be refreshed.

The results in HOP-62 cells indicate that CCK-2R inhibition delays sphere growth and may interfere in sphere maintenance too. Up until day 4 the YM022 group showed less sphere growth compared to the others, but after day 5 sphere measurements in all three groups became similar, illustrating that the YM022-treated cells formed spheres very quickly between day 4 and 5, just after YM022 was replenished. In the glioma cell-line, U251, CCK-2R inhibition does not affect sphere maintenance but does reduce the ability to form spheres. These findings suggest that the CCK-2R may be involved in tumour expansion. Results in the colorectal cell-lines, HCT116, HT29 and cisplatin-resistant HCT116, showed that YM022 treatment caused no significant differences in sphere number or area over time. The monolayer cells treated with YM022 at the same time did not show any effects.

YM022 was added at intervals of 72 hours, since this was when the growth factors were replenished. The literature does not state a half-life for YM022 however, meaning that it is possible that this time-period would allow for peaks and troughs of CCK-2R signalling. Further investigations *in vitro* and *in vivo* are needed to fully understand whether the changes in sphere formation and maintenance are related to tumour growth or the CSC subset. These could include the creation of inducible knock-out constructs, allowing CCK-2R signalling to be reliably removed during *in vitro* growth studies. Since the gold standard in validating CSC markers is the ability of these cells to produce a tumour *in vivo* this should also be tested, initially by investigating CCK-2R expression in xenograft tumours using LATE-PCR and flow cytometry, comparing it to that of cell-lines and primary tumours. This could be followed up using the knock-out constructs to see whether lack of CCK-2R signalling can inhibit xenograft growth. If molecular probes could be optimised to show the location of the CCK-2R inside these cells, further work could also include finding out where the CCK-2R<sup>hi</sup> cells are within the tumour, and whether this changes over time.

## 6.5. Conclusions

The CCK-2R is an important receptor in the carcinogenic process. In this project it has been shown to be expressed, at the gene level, in cancer cells of a number of different origins, by a new, highly sensitive, real-time PCR method; LATE-PCR. Expression has been demonstrated, where previously it was thought not to occur, for example in NSCLC and glioblastoma cells. Gene level detection of critical cancer biomarkers is vital in order to better understand how these proteins are made and used in the initiation and progression of this disease. Now that LATE-PCR has been used to this purpose, it could also be applied to discover whether there are other low-level expression mRNAs which are critical in cancer biology.

CCK-2R has long been known to be up-regulated at the protein level in primary tumour cells; however, in this project gene expression levels were shown to be elevated in lung tumours compared to equivalent normal samples. If we are to find out whether CCK-2R is a therapeutic marker in lung cancer, investigating the regulation of the CCK-2R is clearly important. This includes transcriptional regulation, where the 500bp of the promoter proximal to the CCK-2R gene are highly active in several different cancer cell-lines, and post-transcriptional or translational regulation since mRNA and protein levels do not correlate.

CCK-2R was expressed at a high level in a subset of each cell-line population, but the lack of correlation between CCK-2R and some CSC markers, indicates that this is not the CSC subset. The rapidly-dividing cells at the outer edge of the sphere show the highest CCK-2R protein expression, not those in the hypoxic centre. CCK-2R gene expression was, however, up-regulated by both 3D culture of cancer cell-lines and drug-resistance in a colorectal cell-line. This project also showed that inhibition of CCK-2R signalling can inhibit sphere growth and possibly

sphere maintenance in lung and glioma cell-lines, suggesting a role for the CCK-2R in tumour expansion.

## Chapter 7    Bibliography

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